RNA based signature for cancer classifications

David Gentien, Head of the Genomics Platform
Translational Research Department
Institut Curie - Research Centre

The Translational Research Dpt:

Head: Sergio Roman Roman, Pharm D
Assist.: Dominique Gallier

Research teams and groups, platforms and expertise (gates): a dynamic toolbox

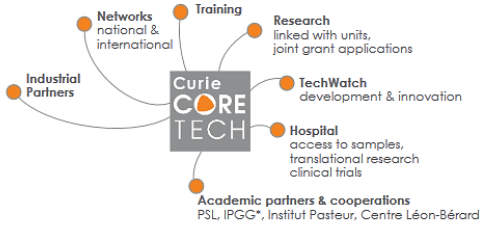
Enabling internal and external (academic & industrial partners) proof of concept projets

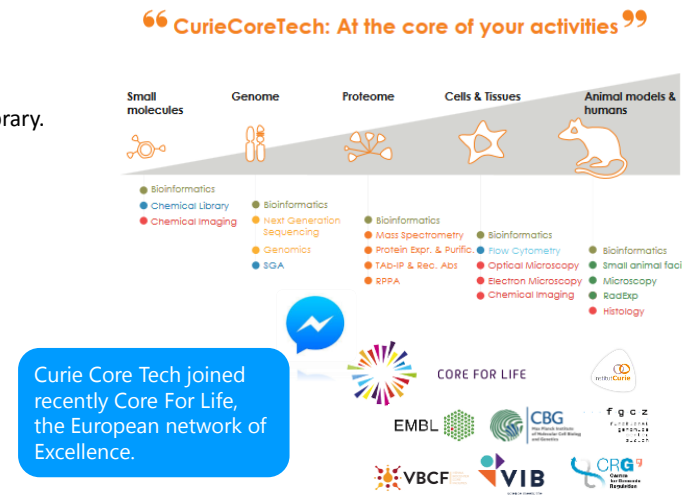
Teams	Translational groups	Translational platforms
FSHR and cancer Nicolae Ghinea, DR1 Inserm		**Genomics** David Gentien, IR Curie
Translational Pediatrics U830 Gudrun Schleiermacher MD-PhD - Curie	**Triple negative breast cancer** Thierry Dubois, manager, IR Curie	**High-content screening** Elaine Del Nery, IR Curie
Resp. rate in breast cancer U932 Fabien Reyal, MD-PhD - Curie	**Circulating biomarkers** Charlotte Proudhon, manager, Curie	**Protein arrays (RPPA)** Leanne de Koning, IR Curie
Immunotherapy U932 Eliane Piaggio, DR2 Inserm Delphine Loirat, MD-PhD - Curie	**Uveal melanoma** Samar Alsafadi, manager, Curie	**Experimental Radiotherapy** Frédéric Pouzoulet, IR Curie
Plasticity & epigenetic UMR 3244 Céline Vallot, CR2 CNRS		**Preclinical Investigation Laboratory (LIP)** Didier Decaudin, MD-PhD - Curie
Integrative functional genomics of cancer Josh Waterfall, PhD - Curie		

Curie Core Tech

Coordinator : Dr Andrea Hutterer

9 technology platforms
5 translational gates
2 in-house services
1 central resource, the chemical library.





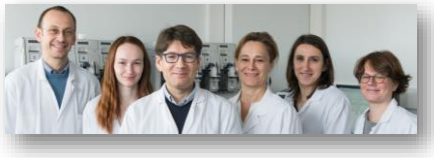
Curie Core Tech joined recently Core For Life, the European network of Excellence.

CORE FOR LIFE

EMBL CBG

VBCF VIB CRG

A Genomics platform dedicated to everyone



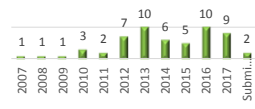
A team composed of 5,8 FTEs:

- Dedicated to project establishment, tailored analysis,
- Involved in the setup of pipelines to fit to clinicians requirements (precision medicine programs)
- Ready for sample preparation up to primary analysis of raw data
- Implicated in the evaluation / comparison of new methods to improve genome analysis (FF, FNA, FFPE, etc.)
- Implicated in the training of young scientists (bachelor, master)

Affymetrix

Nanostring

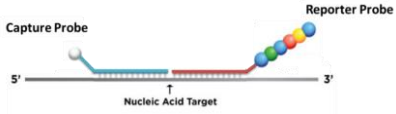
56 publications including almost one member of the platform in authors

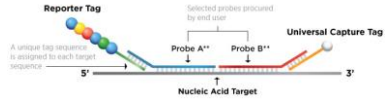


Targeted genomics:
Multiplexed and direct digital counting

- Different possibilities to detect up to 800 nucleic acids targets via specific probe designs

Breast cancer intrinsic subtypes + Medulloblastoma

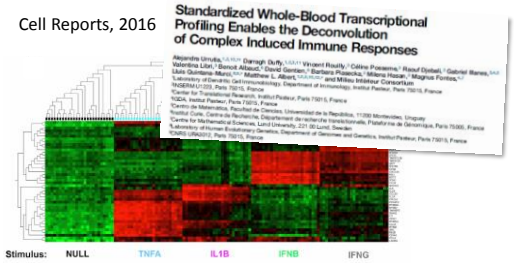


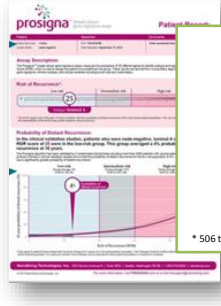


- Easy to use on large cohorts, and easy to transfer into clinical daily practices

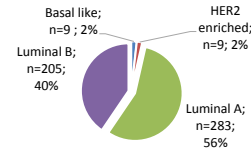
Hierarchical clustering of donors based on the filtered gene list and four cytokine stimuli and Null condition showing the unique and overlapping expression. From Urrutia A. et al.

Cell Reports, 2016





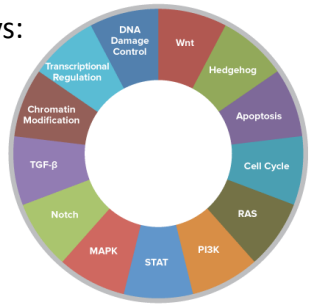
Prosigna tests achieved within a 18 month period*



* 506 tests achieved between January 2016 and June 2017 at Institut Curie.

Vantage 3D Protein Solid Tumor Signaling Content

Cancer Pathways:
(mRNA and proteins)



SNV Panel: 25 genes covering 104 most relevant SNVs and small InDels, which include mutant and reference probe per SNV

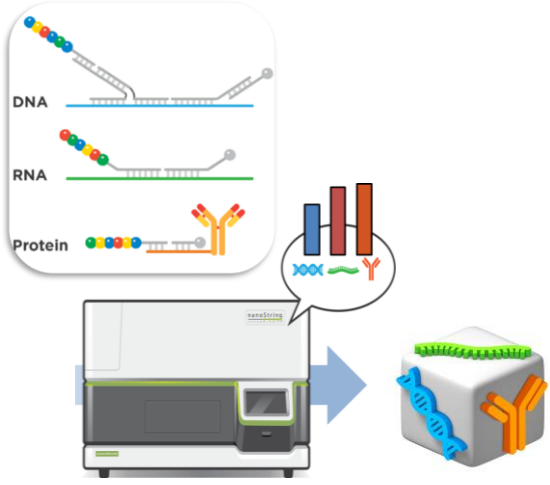
SNV Solid Tumor Panel				
BRAF	KIT	CTNNB1	PTPN11	PIK3CA
EGFR	GNAQ	PTEN	ROS1	FBXW7
KRAS	GNA11	APC	KEAP1	ERBB2
MET	FGFR2	BRCA1	NFE2L2	ALK
NRAS	STK11	BRCA2	TP53	JAK2

Target	Driver Gene	MAPK	PI3K	Ras	Cell Cycle
4E-BP1/Phospho-4E-BP1 (Thr37/46)	-	-	+	-	-
EGF Receptor/Phospho-EGF Receptor (Tyr1068)	+	+	+	+	-
GSK-3β/Phospho-GSK-3β (Ser9)	-	-	+	-	+
HER2/ErbB2	+	+	+	+	-
Ki-67	-	-	-	-	+
Met	+	-	+	+	-
ERK/Phospho-ERK (Thr202/Tyr204)	-	+	+	+	-
p53	+	+	+	-	+
Akt/Phospho-Akt (Ser473)	+	+	+	+	+
Keratin	-	+	-	-	-
Phospho-AMPKα (Thr172)	-	-	+	-	-
Phospho-Chk1 (Ser345)*	-	-	-	-	-
Phospho-c-Raf (Ser259)	-	+	+	+	-
Phospho-Histone H3 (Ser10)	-	-	-	-	+
Phospho-MEK1/2 (Ser217/221)	+	+	+	+	-
Phospho-p70 S6 Kinase (Thr389)*	-	-	+	-	-
Phospho-PDK1 (Ser241)	-	-	+	-	-
Phospho-PRAS40 (Thr246)	-	-	+	-	-
Progesterone Receptor	+	-	-	-	-
S6 Ribosomal Protein/Phospho-S6 Ribosomal Protein (Ser235/236)	-	-	+	-	-
Tuberin/TSC2/Phospho-Tuberin/TSC2 (Thr1462)	-	-	+	-	-

*Phospho-p70 S6 Kinase (Thr389) and Phospho-Chk1 (Ser345) for lysate only

7

Setup of new multiplexed approaches to analyze simultaneously DNA, RNA and proteins

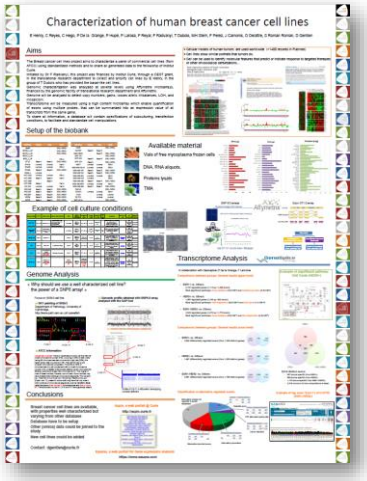


Our motivations:

- ❑ **Can we detect known mutations?**
- ❑ **What are the deregulated cancer pathways after treatment of breast cancer cell lines?**
- ❑ **Is this toolbox properly working on fresh frozen material and formalin fixed and paraffin embedded material and can we validate them by RPPA and/or IHC??**

8

Breast cancer cell lines as models for evaluation of "3D Biology"



Translational Research program initiated in 2009

HCC1954[a]
SKBr3[a]
MDA MB 231[b]
MCF7[c]

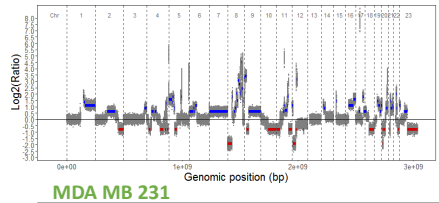
✓ PAM50 classification:
a: Her2+;
b: Basal like;
c: Luminal B

✓ STR analysis:
Authentication confirmed

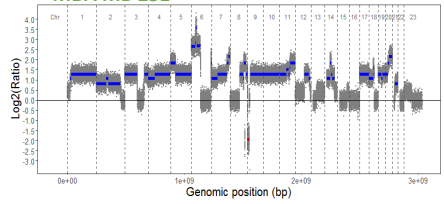
Known genomic mutations:
From COSMIC and ATCC database

Whole Genome Copy Number analysis (Curie dataset)

HCC 19544



MDA MB 231



9

In collaboration with the RPPA and PathEx platforms:

Four different breast cancer cell lines

HCC1954[a]
SKBr3[a]
MDA MB 231[b]
MCF7[c]

PAM50 classification:
a: Her2+;
b: Basal like;
c: Luminal B

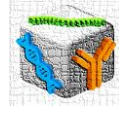
Cultured in 3 different Conditions done in duplicates

W/o any treatment
Selumetinib
Lapatinib

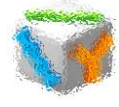
Prepared accorded 2 different ways

FF
FFPE

Specific Nanostring workflow



DNA, RNA and Protein quantification



4

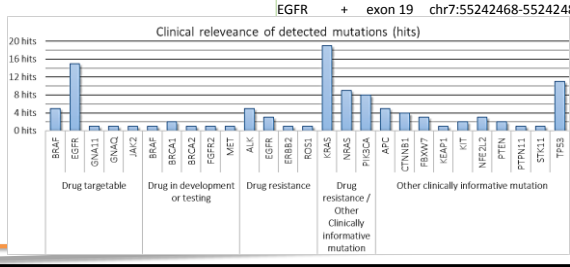
Rapid overview of the SNV panel

- > ***104 driver mutations from 25 key solid tumor genes are analyzed***
- > ***Common cancer-related mutations associated are considered (but can be re-adjusted).***
- > ***Different type of mutations are taken into account***
- > ***Clinical relevance is assigned for each mutation.***

Gene	Exon	COSMIC ID	Pancreatic	Uveal Mel.	Skin	Thyroid	Colorectal	Lung	Uterine	Breast	Cervical	Stomach	Bladder	Esophageal	Brain	Ovarian	Liver	Kidney	Prostate
BRAF	exon 15	COSM476	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	-	+
GNA11	exon 5	COSM5296 9	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GNAQ	exon 5	COSM2875 8	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-
KRAS	exon 2	COSM520	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KRAS	exon 2	COSM521	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NRAS	exon 3	COSM580	+	+	+	+	+	+	+	-	+	+	+	-	+	-	+	+	+
NRAS	exon 3	COSM584	+	+	+	+	+	+	+	+	-	-	+	-	+	+	+	-	-
PIK3CA	exon 10	COSM764	-	+	+	-	+	-	+	+	-	+	+	-	-	-	+	-	-
TP53	exon 5	COSM1073 3	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-

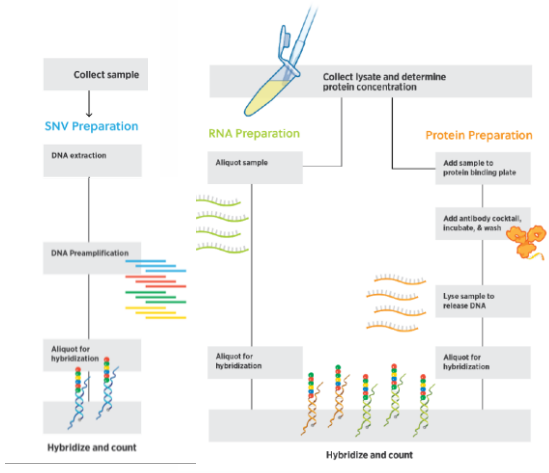
...

Gene	Strand	Exon	Coordinates Hg19	Mutant	Reference	COSMIC ID	mRNA mutation	Protein Mutation
EGFR	+	exon 20	chr7:55249071-55249071	T	C	COSM6240	2369C>T	T790M
EGFR	+	exon 21	chr7:55259515-55259515	G	T	COSM6224	2573T>G	L858R
EGFR	+	exon 18	chr7:55241708-55241708	C	G	COSM6239	2156G>C	G719A
EGFR	+	exon 19	chr7:55242465-55242479	-	GGAATTAAGAGAAGC	COSM6223	2235_2249del15	E746_A750delELREA
EGFR	+	exon 19	chr7:55242466-55242480	-	GAATTAAGAGAAGCA	COSM6225	2236_2250del15	E746_A750delELREA
EGFR	+	exon 19	chr7:55242467-55242483	TTGCT	AATTAAGAGAAGCAACA	COSM12416	2237_2253delTTGCT	E746_T751>VA
EGFR	+	exon 19	chr7:55242467-55242485	T	AATTAAGAGAAGCAACATC	COSM12384	2237_2255delT	E746_S752>V
EGFR	+	exon 19	chr7:55242468-55242483	-	ATTAAGAGAAGCAACA	COSM6254	2239_2253del15	L747_T751delLREAT
				78	C	TTAAGAGAAG	COSM12382	2239_2248delC	L747_A750>P
				86	-	TTAAGAGAAGCAACATCT	COSM6255	2239_2256del18	L747_S752delLREATS
				87	GT	TTAAGAGAAGCAACATCTC	NOCOSM7	2239_2257>GT	L747fs*
				87	-	TAAGAGAAGCAACATCTC	COSM12370	2240_2257del18	L747_P753>S
				31	-	AAC	COSM51525	2127_2129delAAC	E709_T710delinsD
				84	-	TAAGAGAAGCAACAT	COSM12369	2240_2254del15	L747_T751delLREAT
				24	A	T	COSM6213	2582T>A	L861Q

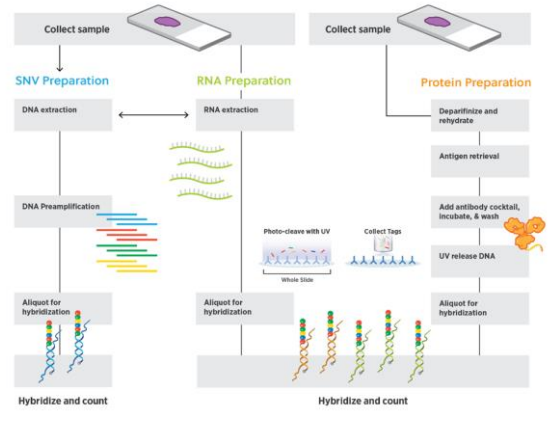


Dedicated workflows to detect DNA, RNA and Proteins

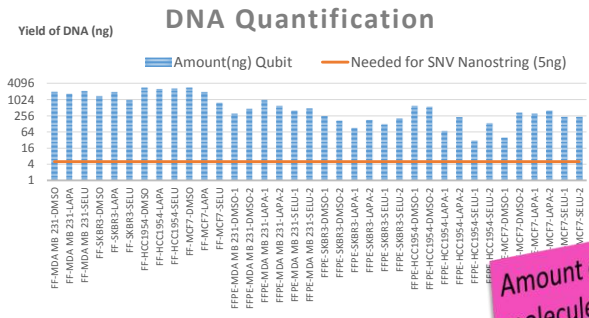
For fresh frozen samples, living cel:



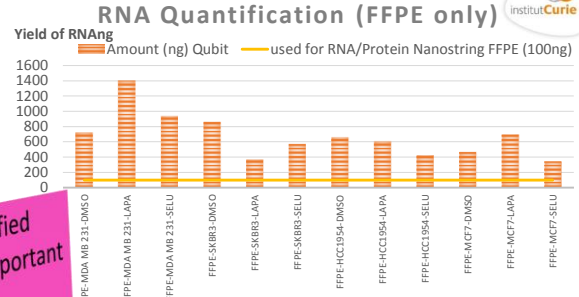
For Formalin fixed and paraffin embedded samples



DNA Quantification

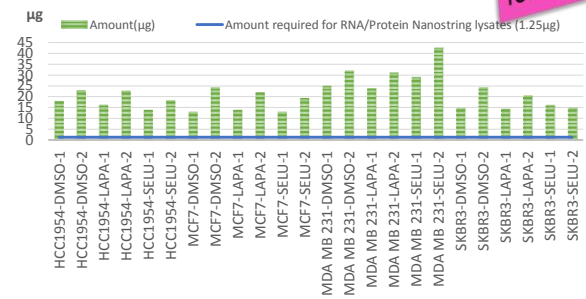


RNA Quantification (FFPE only)



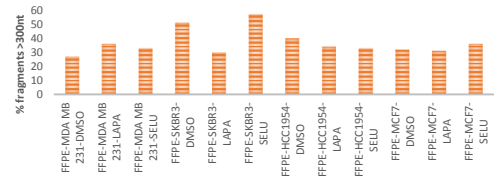
Amount of purified molecules is important for all samples

Protein Quantification (FF only)

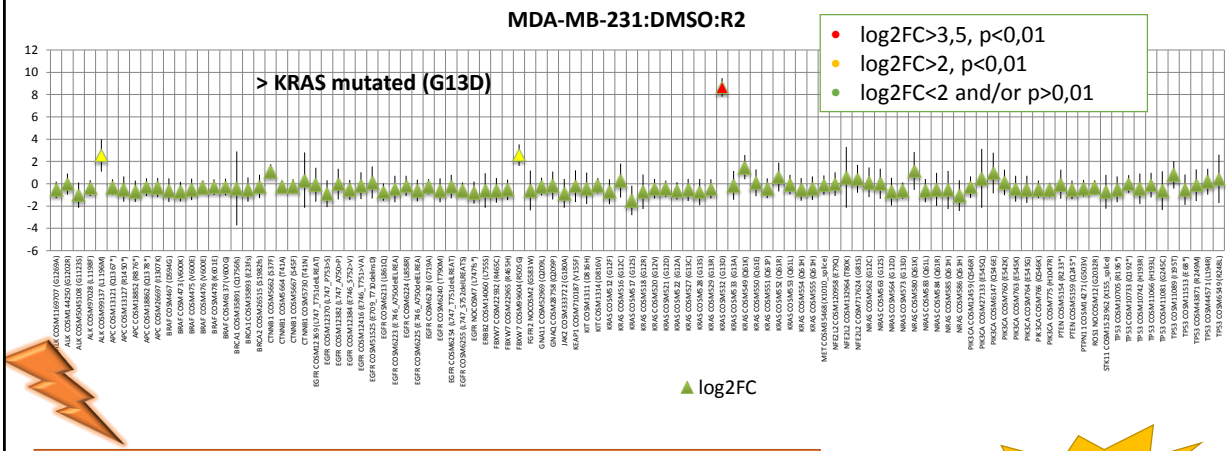


Integrity of total RNA extracted from FFPE

% Fragment >300nt



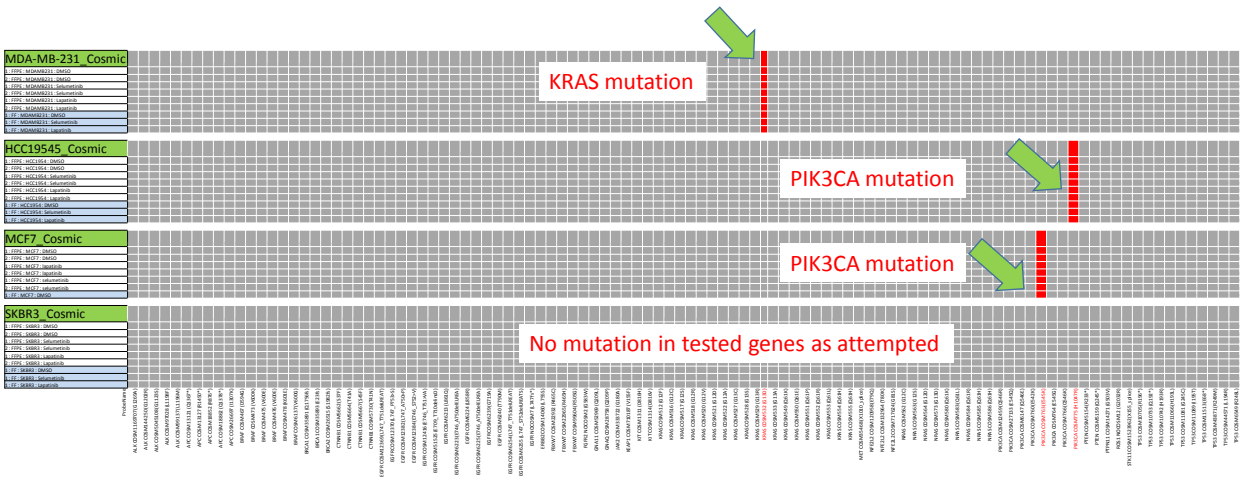
Detection of mutations in MDA MB 231 – FFPE



The detection of mutations requires adjustments of log2FC in FFPE samplesto remove false positive.

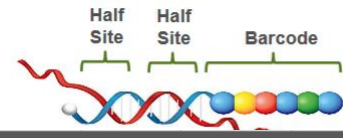
Sensitivity of detection is being evaluated

After adjustment of Log2FC, mutational status are correctly assigned to breast cancer cell lines (whatever the conditions of treatment and preparation of cells)



15

Cancer Pathways Panel (RNA+Protein)



Annotation Summary: RNA vs Protein Probe Coverage

Pathway Annotation	# RNA Probes in panels dedicated to FF/FFPE	# Protein lysates - FF	# Protein Probes - FFPE
Driver Gene	124	9	9
Notch	24	0	0
Wnt	78	3	3
Hedgehog	28	2	2
Chromatin Modification	22	0	0
Transcriptional Misregulation	101	3	3
DNA Damage - Repair	50	1	0
TGF-beta	51	2	2
MAPK	157	11	11
JAK-STAT	86	2	2
PI3K	201	23	22
Ras	142	10	10
Cell Cycle - Apoptosis	137	7	7
Genes of interest (w/o RNA or Protein controls)	770 RNA	24 Protein probes	22 protein probes

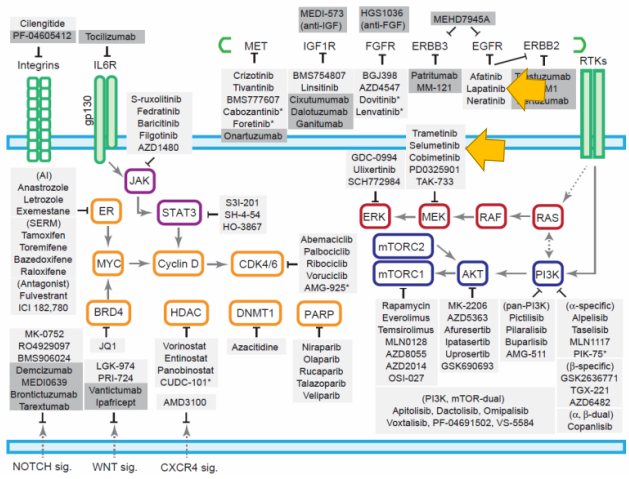
nanoString TECHNOLOGIES /antage 3D™ RNA:Protein Solid Tumor Assay for FFPE - Protein Probe Details

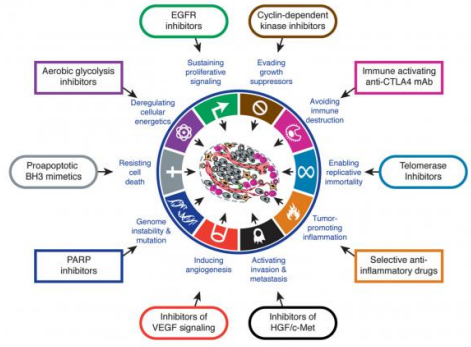
Common Name	HUGO Gene Name	Phosphosite	Uniprot ID	Clone Name
4E-BP1	EIF4EBP1	Total	Q13541	53H11
EGF Receptor	EGFR	Total	P00533	D38B1
GSK-3β	GSK3B	Total	P49841	D5C5Z
HER2/ErbB2	ERBB2	Total	P04626	29D8
Ki-67	MKI67	Total	P46013	8D5
Met	MET	Total	P08581	D1C2
p44/42 MAPK (Erk1/2)	MAPK1 / MAPK3	Total	P27361	137F5
p53	TP53	Total	P04637	DO-7
Pan-Akt	AKT1 / AKT2 / AKT3	Total	P31749	C67E7
Pan-Keratin	KRT4/KRT5/KRT6/KRT8/KRT10/KRT13/KRT18	Total	P13645	C11
Phospho-4E-BP1	EIF4EBP1	Thr37/46	Q13541	236B4
Phospho-Akt	AKT1 / AKT2 / AKT3	Ser473	P31749	D9E
Phospho-AMPKα	PRKAA1 / PRKAA2	Thr172	Q13131	40H9
Phospho-c-Raf	RAF1	Ser259	P04049	Polyclonal
Phospho-EGF Receptor	EGFR	Tyr1068	P00533	D7A5
Phospho-GSK-3β	GSK3A / GSK3B	Ser9	P49841	D85E12
Phospho-Histone H3	HIST1H3A	Ser10	P68431	D2C8
Phospho-MEK1/2	MAP2K1 / MAP2K2	Ser217/221	Q02750	41G9
Phospho-p44/42 MAPK (Erk1/2)	MAPK1 / MAPK3	Thr202/Tyr204	P27361	D13.14.4E
Phospho-PDK1	PDPK1	Ser241	O15530	C49H2
Phospho-PRAS40	AKT1S1	Thr246	Q96B36	D4D2
Phospho-S6 Ribosomal Protein	RPS6	Ser235/236	P62753	D57.2.2E
Phospho-Tuberin/TSC2	TSC2	Thr1462	P49815	5B12
Progesterone Receptor	PGR	Total	P06401	YR85
S6 Ribosomal Protein	RPS6	Total	P62753	54D2
Tuberin/TSC2	TSC2	Total	P49815	D93F12
Protein Controls				
Histone H3-control	HIST1H3A	Total (Positive Contr	P68431	D1H2
Mouse IgG1 control	N/A	N/A (Negative Control)		MOPC-21
Rabbit IgG control	N/A	N/A (Negative Control)		DA1E

 LBL-10370-02

16

Interest in RTKs dependent pathways, its targeting, in downstream activations

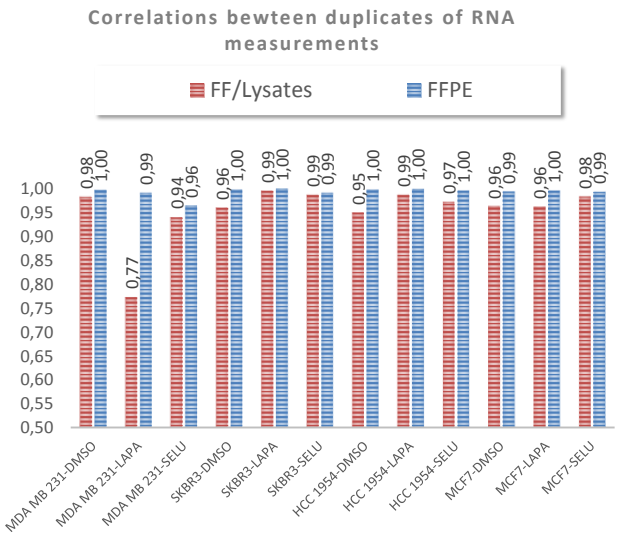


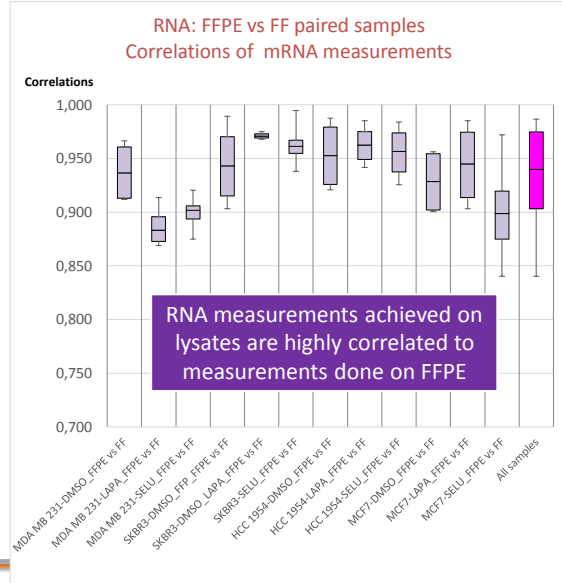


Jin and Mu. Targeting Breast Cancer Metastasis. *Breast Cancer: Basic and Clinical Research* 2015:9(S1) 23–34 doi:10.4137/BCBCR.S25460.

17

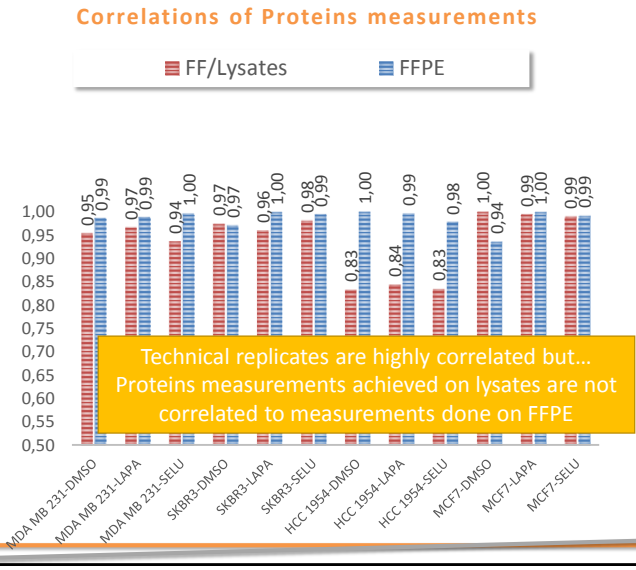
Comparisons of 3D Biology PanCancerPathway Panel : mRNA

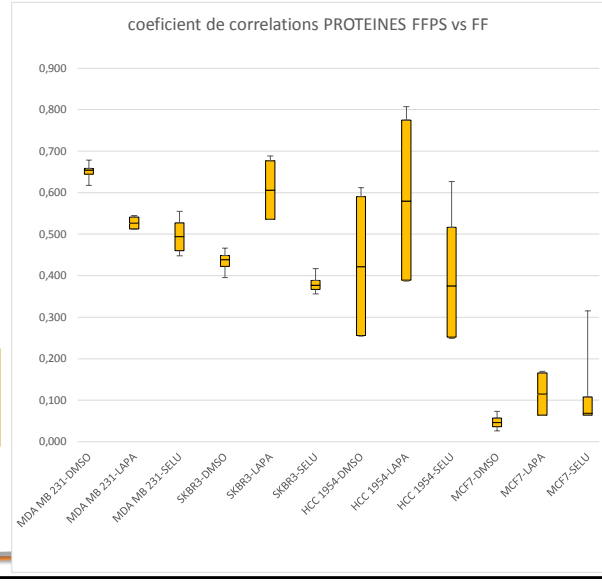




18

Comparisons of 3D Biology PanCancerPathway Panel : Proteins

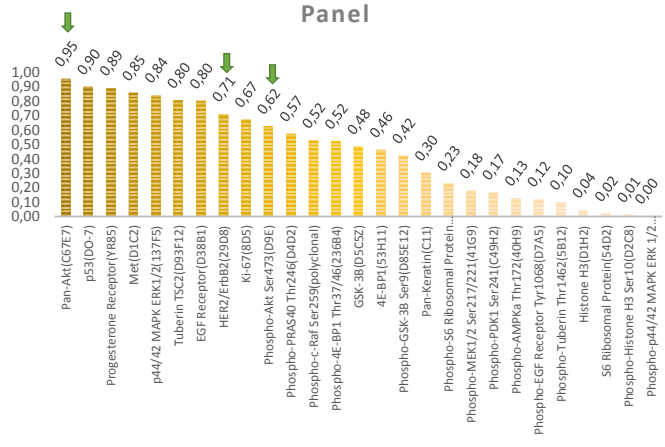




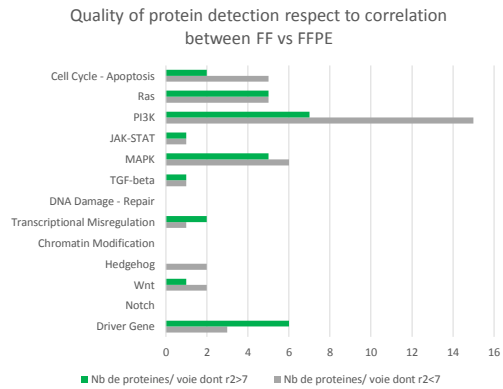
19

Performance of protein detection is varying between FFPE and Fresh frozen Lysates

Protein measurements in FF vs FFPE samples via the Nanostring PanCancer Panel

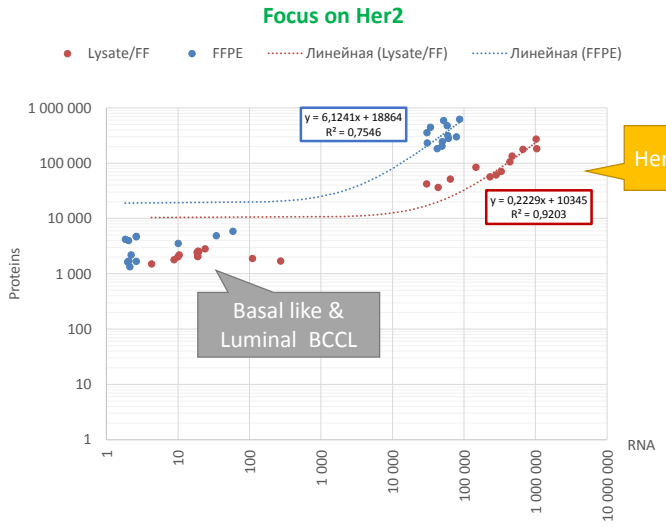


8 proteins out of 27 are similarly detected in the 2 preparation methods



20

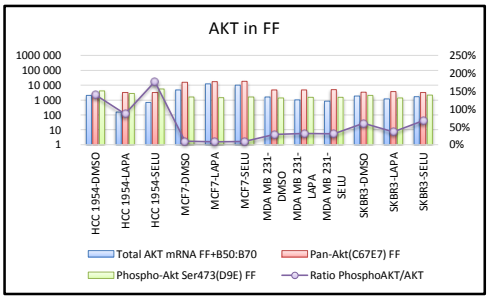
Comparisons of 3D Biology PanCancerPathway Panel : RNA vs Proteins

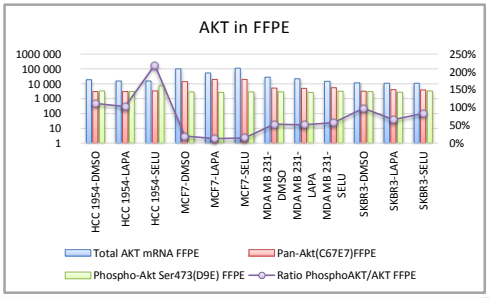


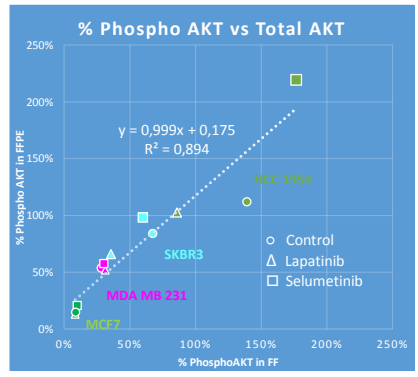
- Range of mRNA/Protein detection is specific to sample preparation
- Tendencies of RNA-Protein detection are preserved across multiple preparation procedures
- The RNA/protein detection seems to follow a linear or plateau detection mode within a single preparation procedure

21

Comparisons of 3D Biology PanCancerPathway Panel : RNA, Proteins and Phospho Proteins







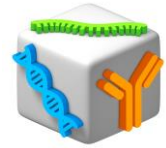
- Percentage of AKT phosphorylation is reproducible between FF and FFPE experiments
- In Her2 positive cell lines (HCC1954), AKT pathway activation is altered after Lapatinib treatment (RTK blockade), or increased after Selutmenib (MEK inhibition).

22

Conclusions

Can we detect known mutations?

- ✓ In FF/lystate: Yes
- ✓ In FFPE's: Yes but adjustments are mandatory
- ❑ Sensitivity of detection is under evaluation

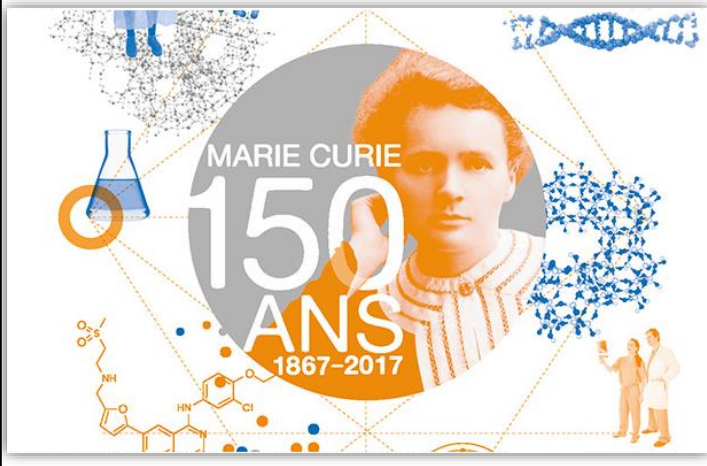


What are the deregulated cancer pathways after treatment of breast cancer cell lines?

- ✓ RNA quantifications are promising (correlations) in FF and FFPE
- ❑ In FF/lystate and in In FFPEs: analysis is on going
- ✓ Proteins measurements in FF are promising (reproducibility and correlation to mRNA).
- ❑ Proteins measurements in FFPE need improvements (antigen retrieval, antibody recognition)

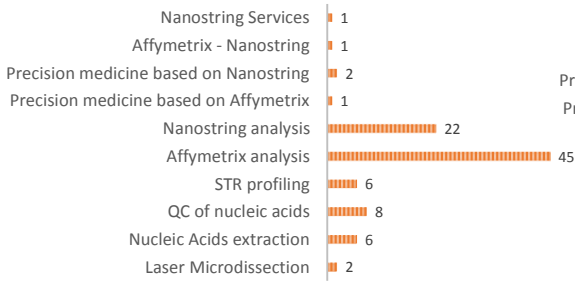
Is this toolbox properly working on fresh frozen material and formalin fixed and paraffin embedded material and can we validate them by RPPA and/or IHC?? No answer yet

23

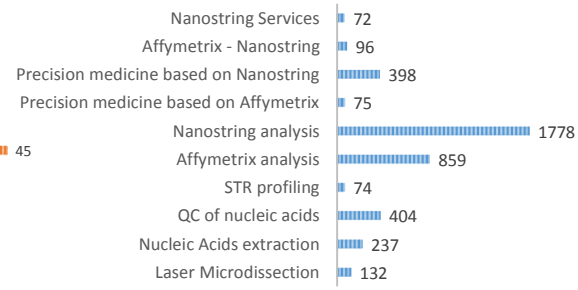


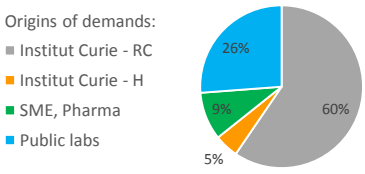
Overview of demands received since January 2017

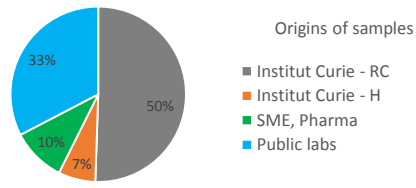
> 94 different analysis submitted



> Concerning 4125 Samples







Acknowledgments

Genomics Platform: Audrey Rapinat, Emilie Henry, Nicolas Fort, Romain Lavigne, Benoit Albaud, Cécile Reyes, Aude Vieillefon.

RPPA Platform: Berengere Ouine, Leanne De Koning.

Pathex Platform: Renaud Leclere, André Nicolas, Didier Meseure.

Nanostring: Rudy van Eijsden , Serge Scherrer , Joel Nelson.

27

Medulloblastoma classification based on Nanostring tools: Dr Julien Masliah Planchon, Dr Franck Bourdeaut, MD,PhD

28

Signature « Northcott »

Acta Neuropathol (2012) 123:615–626
DOI 10.1007/s00401-011-0899-7

METHODS PAPER

Rapid, reliable, and reproducible molecular sub-grouping of clinical medulloblastoma samples

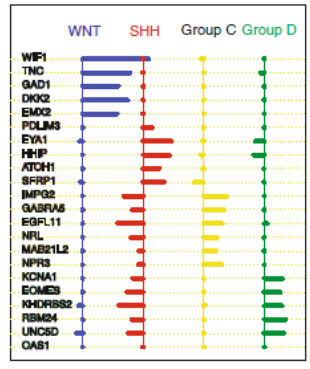
Paul A. Northcott · David J. H. Shih · Marc Remke · Yoon-Jae Cho · Marcel Kool · Cynthia Hawkins · Charles G. Eberhart · Adrian Dubuc · Toumy Guettouche · Yoslayma Cardentey · Eric Bouffet · Scott L. Pomeroy · Marco Marra · David Malkin · James T. Rutka · Andrey Korshunov · Stefan Pfister · Michael D. Taylor

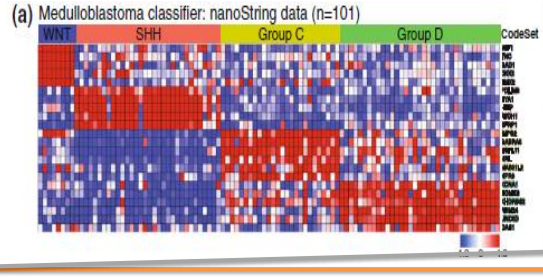
Received: 29 September 2011 / Revised: 19 October 2011 / Accepted: 21 October 2011 / Published online: 6 November 2011

Abstract The diagnosis of medulloblastoma likely encompasses several distinct entities, with recent evidence for the existence of at least four unique molecular subgroups that exhibit distinct genetic, transcriptional, demographic, planning and execution of medulloblastoma clinical that stratify by subgroup, or which are targeted to a sp subgroup requires technologies that can be economi rapidly, reliably, and reproducibly applied to formalin

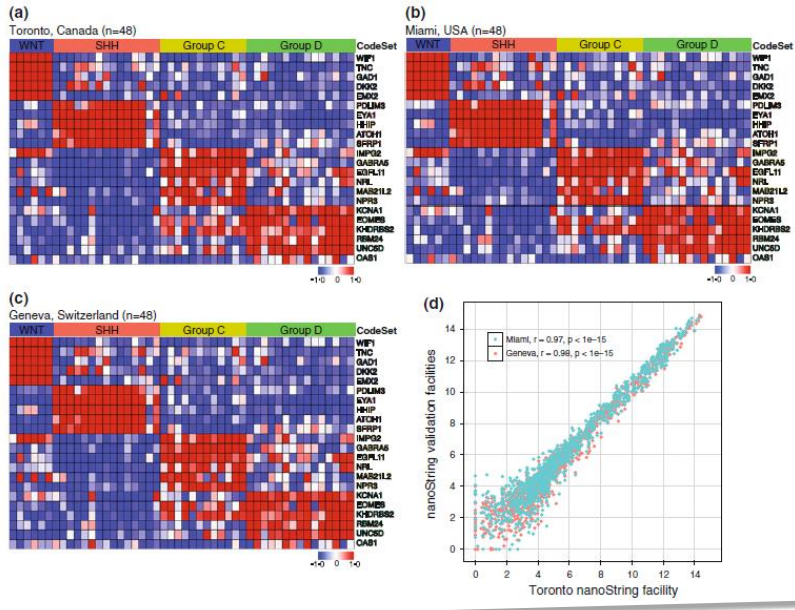
Codeset « Northcott »

Gene symbol	Accession	Gene description	Cytoband	Subgroup-specific fold-change
WNT				
WIF1	NM_007191	WNT inhibitory factor 1	12q14.3	236.4
TNC	NM_002160	tenascin C	9q33	65.9
GAD1	NM_000817	glutamate decarboxylase 1 (brain, 67 kDa)	2q31	63.2
DKK2	NM_014421	dickkopf homolog 2 (*Xenopus laevis*)	4q25	55.9
EMX2	NM_004098	empty spiracles homeobox 2	10q26.1	44.7
SHH				
PDLIM3	NM_014476	PDZ and LIM domain 3	4q35	32.1
EYA1	NM_172059	eyes absent homolog 1 (*Drosophila*)	8q13.3	20.8
HHIP	NM_022475	hedgehog interacting protein	4q28–q32	19.9
ATOH1	NM_005172	atonal homolog 1 (*Drosophila*)	4q22	15.6
SFRP1	NM_003012	secreted frizzled-related protein 1	8p12–p11.1	15.5
Group C				
IMPG2	NM_016247	interphotoreceptor matrix proteoglycan 2	3q12.2–q12.3	15.1
GABRA5	NM_000810	gamma-aminobutyric acid (GABA) A receptor, alpha 5	15q11.2–q12	14.6
EGFL11	NM_198283	eyes shut homolog (*Drosophila*)	6q12	13.4
NRL	NM_006177	neural retina leucine zipper	14q11.1–q11.2	11.5
MAB21L2	NM_006439	mab-21-like 2 (*C. elegans*)	4q31	10.9
NPR3	NM_000908	natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)	5p14–p13	8.2
Group D				
KCNA1	NM_000217	potassium voltage-gated channel, shaker-related subfamily, member 1 (episodic ataxia with myokymia)	12p13.32	16.4
EOMES	NM_005442	eomesodermin	3p21.3–p21.2	13
KHDRBS2	NM_152688	KH domain containing, RNA binding, signal transduction associated 2	6q11.1	10.8
RBM24	NM_153020	RNA binding motif protein 24	6p22.3	10.7
UNC5D	NM_080872	unc-5 homolog D (*C. elegans*)	8p12	10.7
OAS1	NM_016816	2′,5′-oligoadenylate synthetase 1, 40/46 kDa	12q24.1	10.5

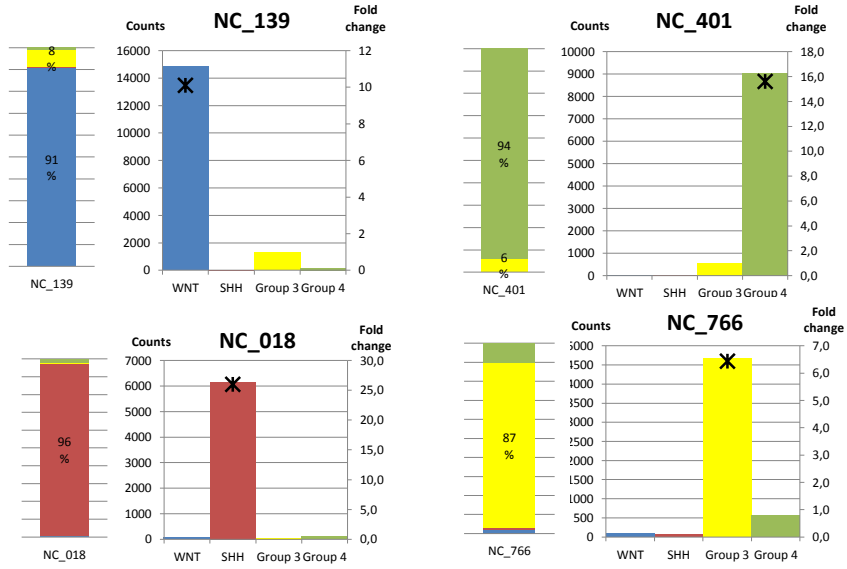




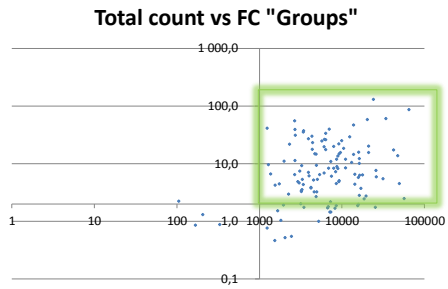
Reproducibility of Northcott's signature

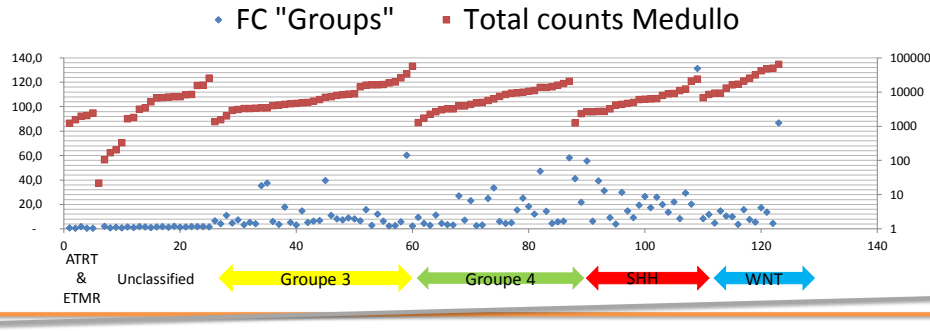


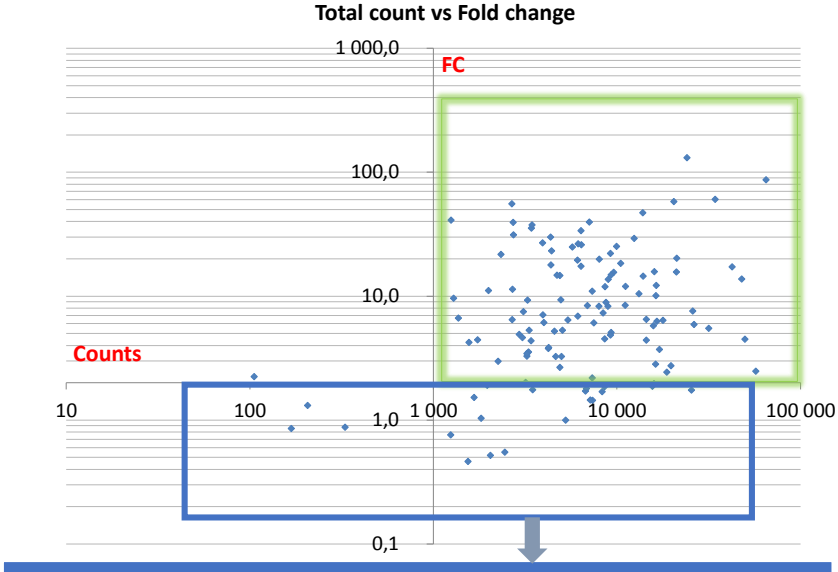
Exemples of medulloblastoma



Groupe	Nombre	Fréquence
Medullo - SHH	22	18%
Medullo - WNT	14	11%
Medullo - Groupe 3	35	28%
Medullo - Groupe 4	27	22%
AT/RTs	4	3%
ETMR	1	1%
Unclassified	19	15%
H2O	1	1%
Total général	**123**	**100%**



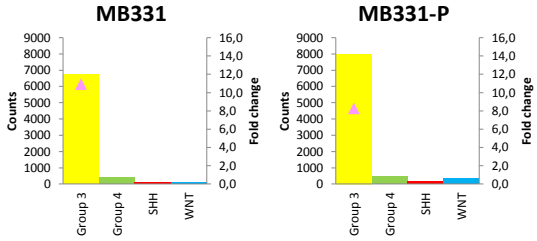




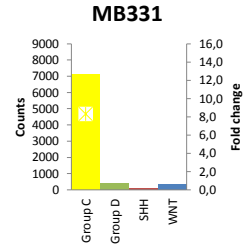
Comparison of codeset lots

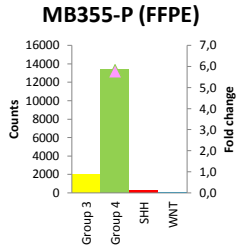
Lots: C2600 and C3326

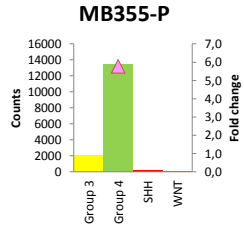
Codeset : C2600



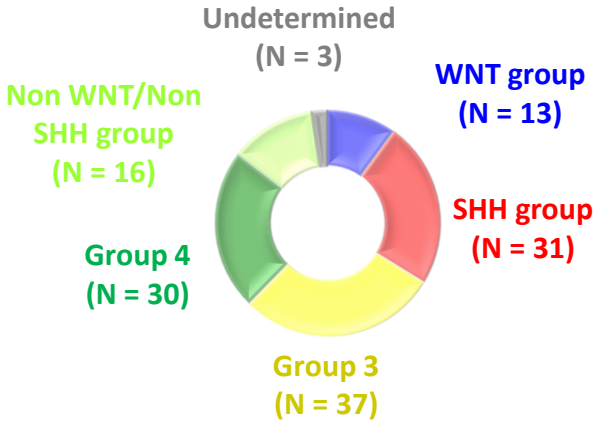
Codeset : C3326



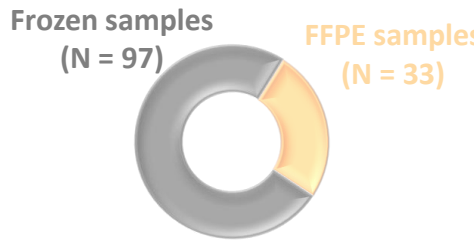




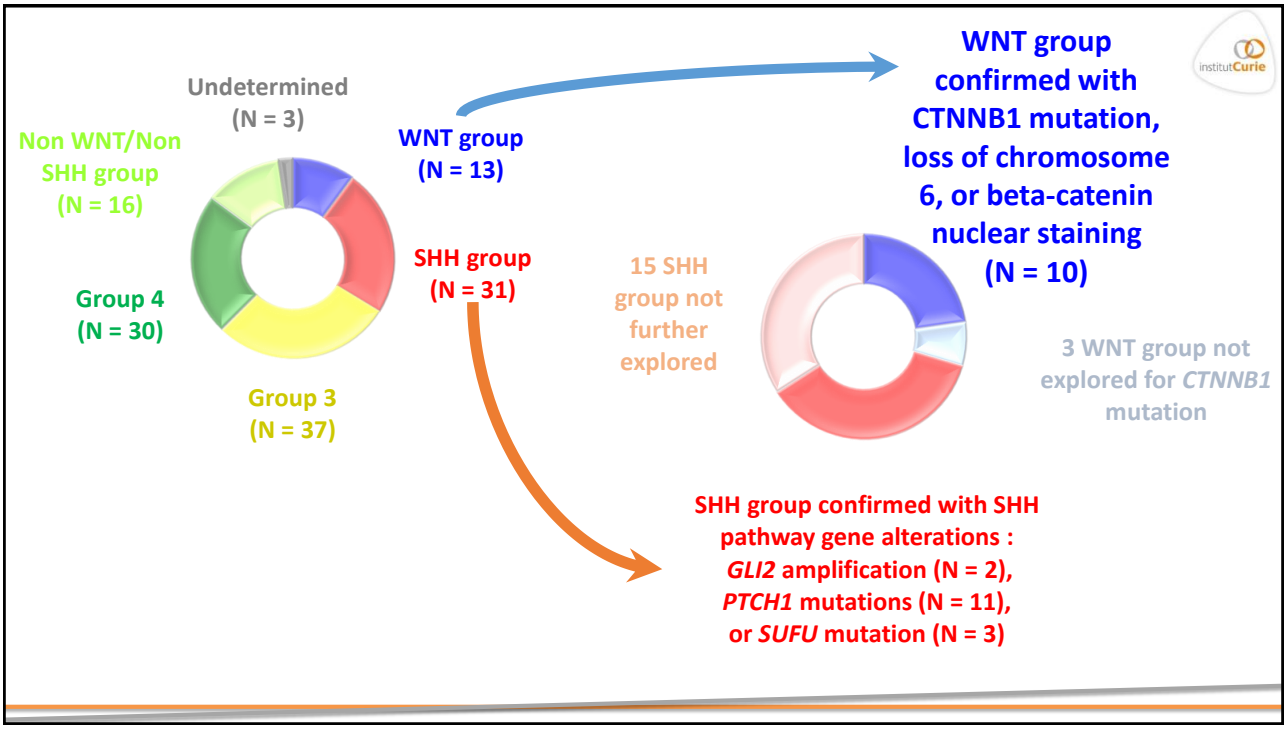
First data collected from Curie: 130 biopsies analyzed



Suitable for low quality RNA



Confirmation of MB groups with subgroup-specific genes sequencing



Cross validation with a methylation-based grouping strategy

Among the 130 medulloblastomas:

- 36 cases were cross-validated with a custom methylation-based Sequenom assay.
  - 32 cases matched perfectly.
  - 3 cases were classified in group 3 with one technology and in group 4 with the other.
  - 1 case that did not match between Nanostring and Sequenom technologies had a low confidence score with Sequenom technology. This cases were classified as WNT group with the Nanostring technology and harbored a *CTNNB1* mutation.

Conclusions

- The NanoString technology represents a simple, rapid, reliable, and cost-effective method to subgroup medulloblastomas that can be used on poor-quality RNA.
- We emphasize that this expression based classification molecular subgrouping integrated with the copy number and mutation profiling of medulloblastoma can be used to improve clinical management and or for future medulloblastoma clinical trials.

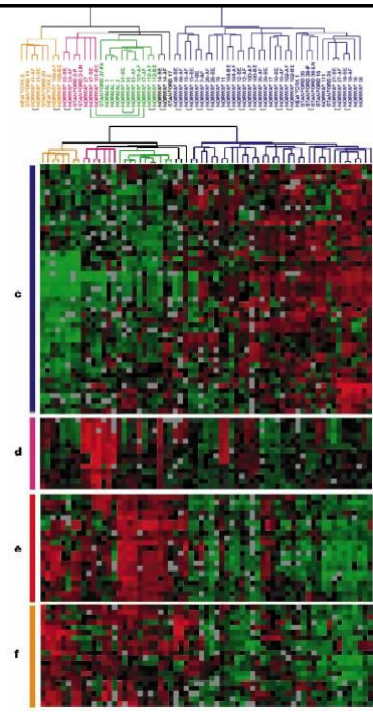
Breast Cancer classification and Prosigna

Molecular portraits of human breast tumours.

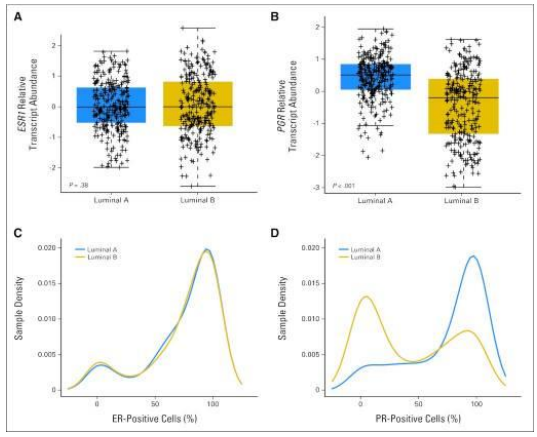
Perou et al. Nature 2000.

- Set of genes from microarrays
- Unsupervised clustering
- 4/5 subtypes of breast cancer
  - Basal-like
  - ERBB2+
  - Luminal A
  - Luminal B

Most of studies are using this classification



Prognostic significance of progesterone receptor-positive tumor cells within immunohistochemically defined luminal A breast cancer.



Prat et al. J Clin Oncol. 2013; 10; 31(2): 203–209.

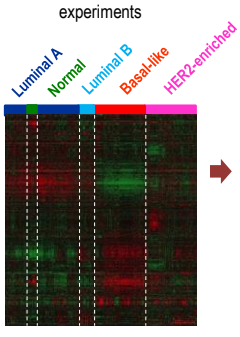
Development of Prosigna is Based on PAM50 Gene Signature

2000
Researchers first describe breast cancer intrinsic subtypes based on microarray experiments

2009
Researchers first describe "PAM50" gene expression signature

2010
NanoString exclusively licenses PAM50 gene expression signature

2013
Prosigna launches after receiving FDA 510k clearance in US and CE Mark for Europe and Israel



Endorsed in 2013 St. Gallen Guidelines [2]

Luminal A	Endocrine therapy alone
Luminal B	If HER2-, endocrine +/- cytotoxic therapy If HER2+, cytotoxics + anti-HER2 + endocrine Could include anthracyclines and taxanes
HER2 enriched	Cytotoxics + anti-HER2 Could include anthracyclines and taxanes
Basal-like	Cytotoxic therapy alone, potentially including anthracyclines, taxanes, and alkylating agent Do not routinely use cisplatin or carboplatin

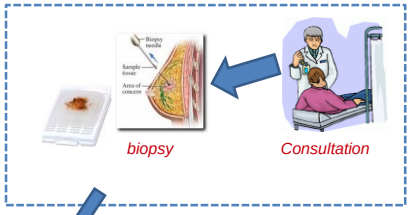
prosigna
Breast cancer prognostic gene signature assay

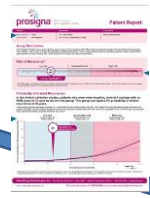
demic breast cancer experts
of North Carolina
ty School of Medicine
gist, BC Cancer Agency
Huntsman Cancer Institute

Source: *Molecular portraits of breast cancer.* Nature. 2000 May 25;.
Source: *Supervised Risk Predictor of Breast Cancer Based on Intrinsic Subtypes, JCO.2009*

44

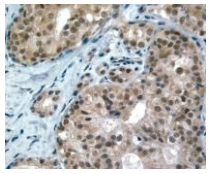
A dedicated organization for personalized medicine using the test Prosigna Pam50





1. DI Study
2. Neopal Trial
3. "PAIR Heterogeneity" research program
4. Genomic Testing proposed by complementary health insurance(s)
5. …

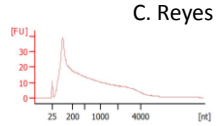
Pathology dept,
Anne Vincent Salomon,



Pharmacogenomic unit
Ivan Bieche
Celine Calens,

RNA extraction

Genomic Platform
D Gentien
B Albaud
E. Henry
A. Vieillefon
A. Rapinat
C. Reyes



prosigna Breast cancer prognostic gene signature assay

45

Three Elements of the Prosigna™ Assay

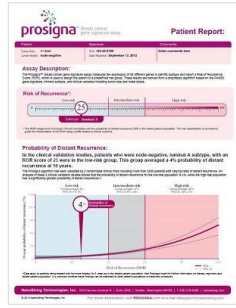
Hardware: nCounter Analysis System[1]	**Consumables:** Prosigna Kits (CE Mark Version)	**Software:** Prosigna Report (CE Mark Version)[2]

Prep Station

Digital Analyzer

Includes:

- 50 gene-based CodeSet with 8 controls
- Other consumables required for assay



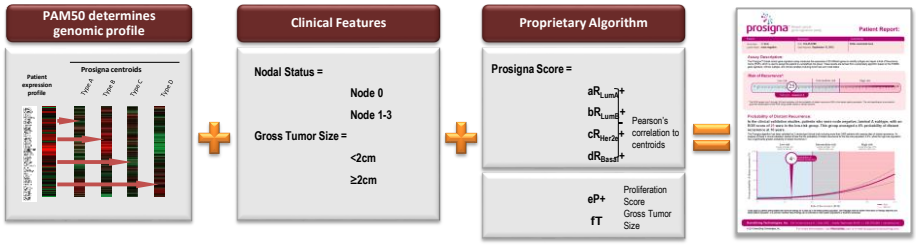
1 The nCounter Analysis System is for research use only in markets that do not recognize the CE Mark and in which Prosigna is not registered.
2 The Prosigna report shown is the version available in markets which recognize the CE Mark. If the FDA clears Prosigna for sale in the U.S., the report will be different from the CE Mark version. For example, output of the U.S. version of Prosigna submitted for 510(k) will not report intrinsic subtype. FDA has advised that reporting intrinsic subtype in the U.S. will require a future PMA supported by additional clinical studies.

NOTE: Please see "Regulatory Information" for additional information on the regulatory status of Prosigna

46

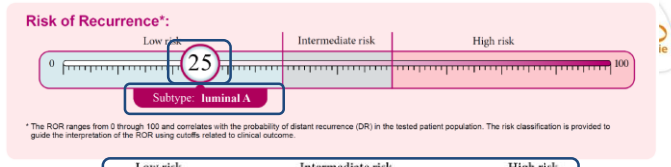
Prosigna Individual Results are Based on Clinical and Genomic Information

- The molecular subtype identified by PAM50 (not reported)
- A proliferation score
- Clinical features of tumor size and nodal status
- Number between 0 - 100

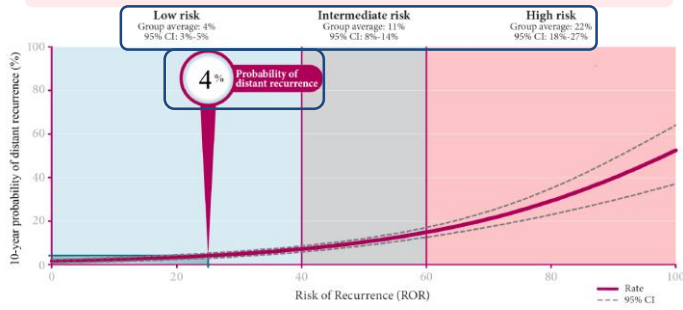


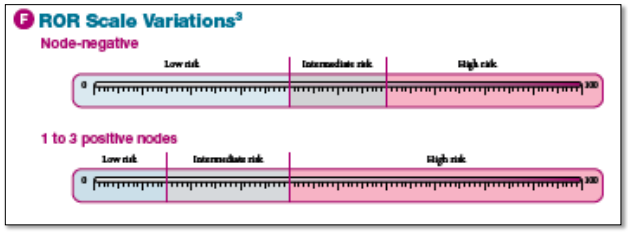
Risk Category:
Specific to your patient's nodal status
Validation set of > 2400 postmenopausal women with early-stage breast cancer

47



Prosigna™ Report





Nodal status	ROR range	Risk categorization
Node-negative	0-40	Low
	41-60	Intermediate
	61-100	High
Node-positive (1-3 nodes)	0-15	Low
	16-40	Intermediate
	41-100	High

Specimen Attribute	Requirement
Type sample	Breast carcinoma (canalaire, lobulaire ou mixte)
Format of sample	FFPE sections of 10 microns
Minimum size of the tumor	4mm^2
Cellularity min	10% within tumor area
Amount of tissue	Area >100mm^2 = 1 section 4mm^2 < tum. Area < 100mm^2 = 3 sections

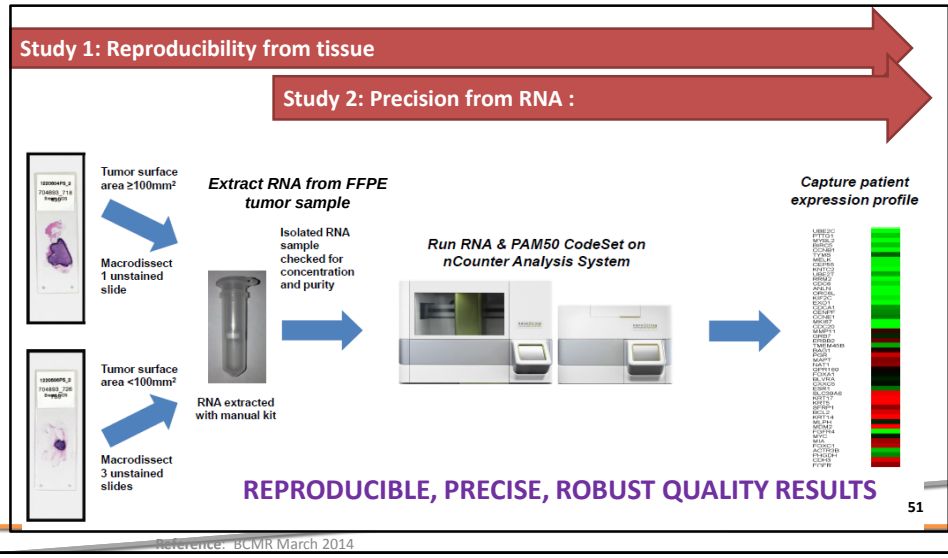
49

Up to 10 samples can be analyzed onto a single cartridge

Steps	Specifications
Minimum amount of RNA	125ng (12.5ng/µl)
Number of analysis per run	10 samples, 2 controls Regsitration of runs throught a webpage or with the nCounter
Hybridization	Over night incubation

Analytic Reproducibility & Precision & Robustness of Prosigna™ Signature Assay Evaluated in Two Studies

Validation studies were designed to measure the analytical robustness of the test across three clinical testing sites

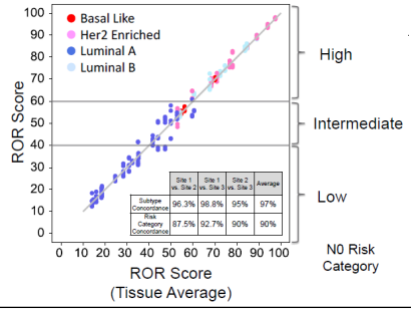


51

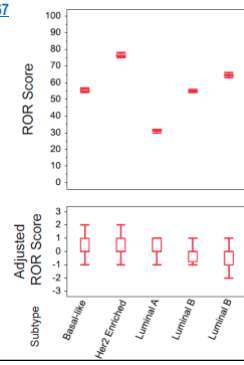
Reference: BCMR March 2014

Analytic Validation Results

- **Study 1: Reproducibility from Tissue**
  - 43 FFPE blocks replicates tissue samples across 3 differents sites and ncounter platforms
  - **Each RNA was tested twice in separate runs**
  - The differences on average between the sites were negligible (<1% total variance).
  - Total ROR standard deviation was 2.89 ROR unit



- **Study 2: Precision from RNA**
  - 108 replicates of each of 5 pooled Breast Tumor samples,
  - **3 different sites , 2 operators at each site, each with 3 different reagents lots, so 9 runs per operator**
  - 100% concordance between the subtype result
  - 100% concordance between risk group
  - Site-to-site or operator-to-operator <1% of variance
  - ROR SD = 0.67



Analytical validation of the PAM50-based Prosigna Breast Cancer Prognostic Gene Signature Assay and nCounter Analysis System using Formalin-fixed paraffin-embedded breast tumor specimens T Nielsen , et al., *BMC Cancer* 2014, 14:177

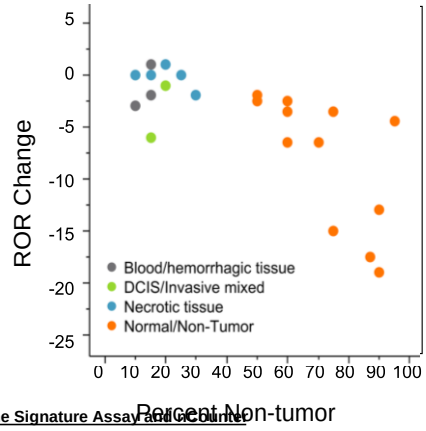
Analytical Reproducibility of the Breast Cancer Intrinsic Subtyping Test and nCounter® Analysis System Using Formalin-Fixed Paraffin-Embedded (FFPE) Breast Tumor Specimens
T Nielsen et al. Poster US CAP 2013

52

52

Prosigna™ Assay Are Robust Against Non-tumor Tissue

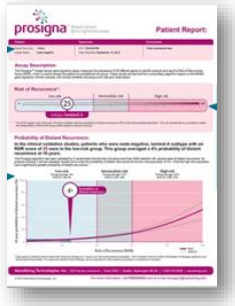
- Objective :
  - Assess impact of adjacent non-tumor tissue on ROR.
- Design:
  - Slide mounted sections from 23 FFPE blocks were tested with vs. without macrodissection of adjacent non-tumor tissue.
  - The difference in ROR between the macrodissected vs. unmacrodissected tissue was determined.
- Result:
  - **Assay results were stable in the presence of moderate amounts of surrounding non-tumor tissue** (<70% by area).



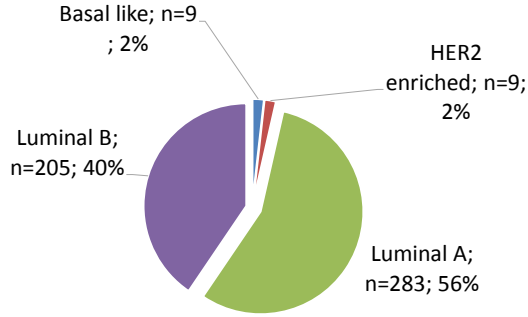
Analytical validation of the PAM50-based Prosigna Breast Cancer Prognostic Gene Signature Assay and nCounter Analysis System using Formalin-fixed paraffin-embedded breast tumor specimens .
Torsten Nielsen Brett Wallden Carl Schaper, Sean Ferree, Shuzhen Liu , Dongxia Gao , Garrett Barry, Naeem Dowidar , Malini Maysuria , James Storhoff , ***BMC Cancer* 2014, 14:177**

Analytical Reproducibility of the Breast Cancer Intrinsic Subtyping Test and nCounter® Analysis System Using Formalin-Fixed Paraffin-Embedded (FFPE) Breast Tumor Specimens. T Nielsen et al., **USCAP 2013**

53



Prosigna tests achieved within a 18 month period*

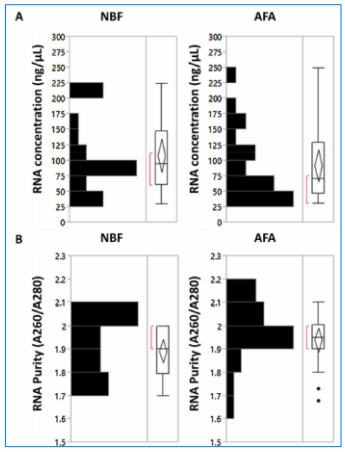


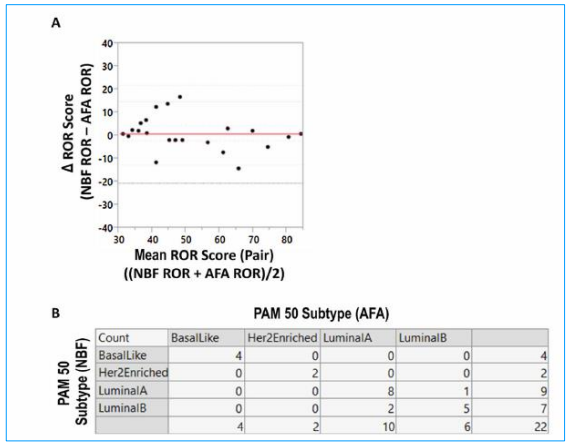
* 506 tests achieved between January 2016 and June 2017 at Institut Curie.

Full Length Article

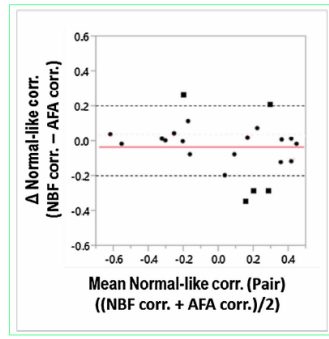
Denaturing fixatives are compatible with the NanoString nCounter® platform and the Prosigna® assay

Roman Rouzier[a,*], Aurelie Roulot[a], Arthur H. Jeiranian[b], Namratha Ram[b], Jean Marc Guinebretiere[c], Anne Vincent Salomon[c], David Gentien[d]





Count	BasalLike	Her2Enriched	LuminalA	LuminalB	
BasalLike	4	0	0	0	4
Her2Enriched	0	2	0	0	2
LuminalA	0	0	8	1	9
LuminalB	0	0	2	5	7
	4	2	10	6	22



55

Our Prosigna *history*

1. **DECISION IMPACT STUDY (PI: Pr Roman Rouzier**): Evaluation of Prospective multi-center study of the impact of the Prosigna® assay on adjuvant clinical decision-making in women with early stage breast cancer. Which patients are the best candidates?

2. **PAIR Heterogeneity (PI: Pr Roman Rouzier):** Can we measure intra-tumor heterogeneity? Spatially and temporary.

3. **Setup of a dedicated pathway for the Genomic test (PAM50)** with the Pathology Dept., the "Pharmacogenomics" unit and the Genomic Platform.

4. Evaluation of the impact of fixative on Prosigna tests (AFA vs Formol).

5. RIHN -> Reimbursement of the test.

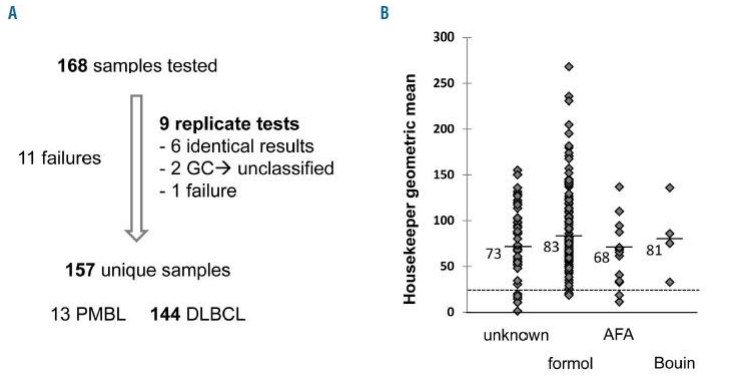
6. Next Steps: **OPTIGENE Trial**. Comparison of 4 genomic tests (Oncotype DX, Mammaprint, Prosigna, IHC4)

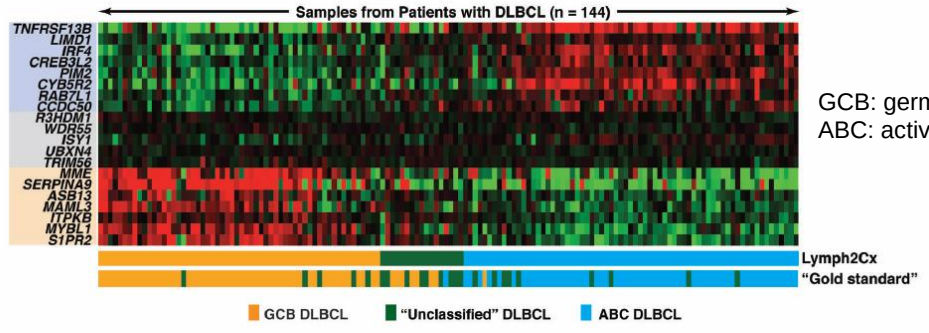
56

Diffuse large B-cell lymphoma classification based on Nanostring tools:
Dr Karen Leroy, LYSARC

Reliable subtype classification of diffuse large B-cell lymphoma samples from GELA LNH2003 trials using the Lymph2Cx gene expression assay

The Lymphoma/Leukemia Molecular Profiling Project (LLMPP) described a digital gene expression-based assay using NanoString technology (Lymph2Cx)

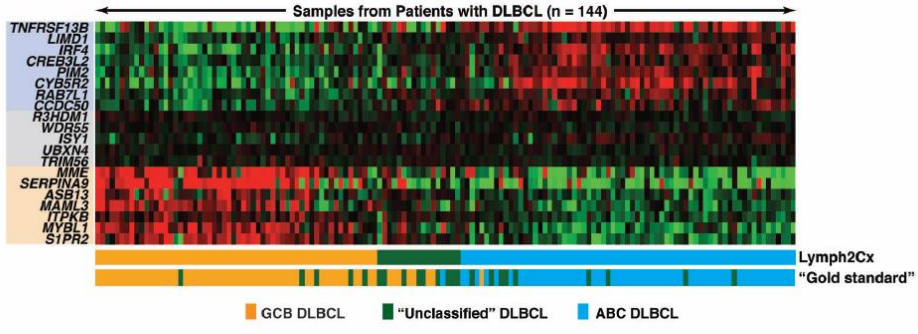




GCB: germinal center B-cell-like
ABC: activated B-cell-like lymphomas

The Affymetrix and Lymph2Cx classifications were concordant in 92.6% (112 of 121) of the cases (91.4% in Scott *et al*.) Samples were :
Unclassified in 6.6.% (8 of 121) (6.9% in Scott *et al*.), or misclassified in 0.8% (1 of 121) (1.7% in Scott *et al*.).

When considering all 3 categories, GCB, ABC and Unclassified, the classifications were concordant in 84% (121 of 144) of the cases (81% in Scott *et al*.), the samples moved from a definitive subtype to Unclassified (or *vice versa*) in 15.3% (22 of 144) (17.6% in Scott *et al*.), and were misclassified in 0.7% (1 of 144) (1.5% in Scott et al.).



9 Affymetrix Unclassified samples were also identified as ABC by the Lymph2Cx assay.

7 (out of 7) The immunohistochemical analysis of these samples showed IRF4 staining (7 of 7 cases with available data).

There was one misclassification, which might correspond to a frozen sample swap, since the FFPE block immunophenotype was CD10 negative, BCL6 negative, IRF4 positive and FOXP1 positive

The major source of discrepancy between the two assays resulted from biopsies, with LPS scores close to the thresholds, shifting between definitive COO subtypes and the Unclassified category. These "intermediate" scores might correspond to samples with low tumor content (as previously reported by Scott *et al*. and observed in 2 cases for which we performed re-extraction and a second analysis), lymphomas with a particular immune infiltrate, or a true "third" DLBCL subtype that has yet to be identified.

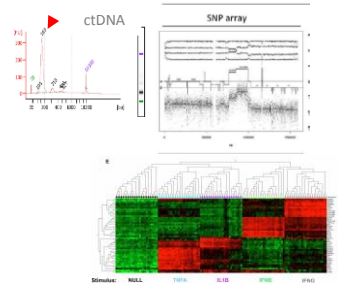
Conclusions

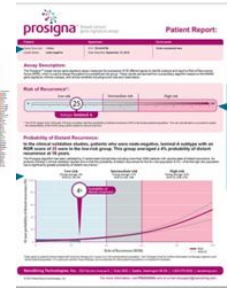
- Gene expression signatures can be translated into clinical practices
- Requires external cohort for validation
- Signatures are working on low integrity RNA
- Results can be generated in short delay (within a week)

GENOMICS (2/3)

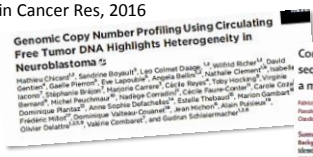
Our capacity

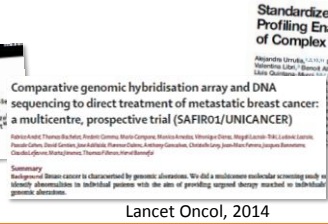
- Analysis of a couple of markers to millions, in single experiments,
- Analysis of a couple of sample to thousands,
  - From single cell to tissue for gene expression,
  - From poor integrity / archived material to high quality material.
  - For rapid or standard analysis,
  - For multiple types of analysis (DNA, RNA, Proteins)
- Share of know how (ctDNA on Oncoscan, Nanostring, Unicancer network and I. Pasteur)



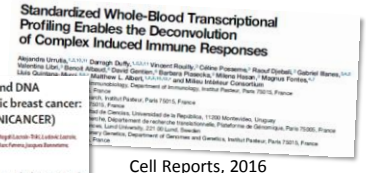


Clin Cancer Res, 2016


Genomic Copy Number Profiling Using Circulating Free Tumor DNA Highlights Heterogeneity in Neuroblastoma

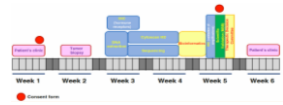

Comparative genomic hybridisation array and DNA sequencing to direct treatment of metastatic breast cancer: a multicentre, prospective trial (SAFIR01/UNICANCER)

Lancet Oncol, 2014

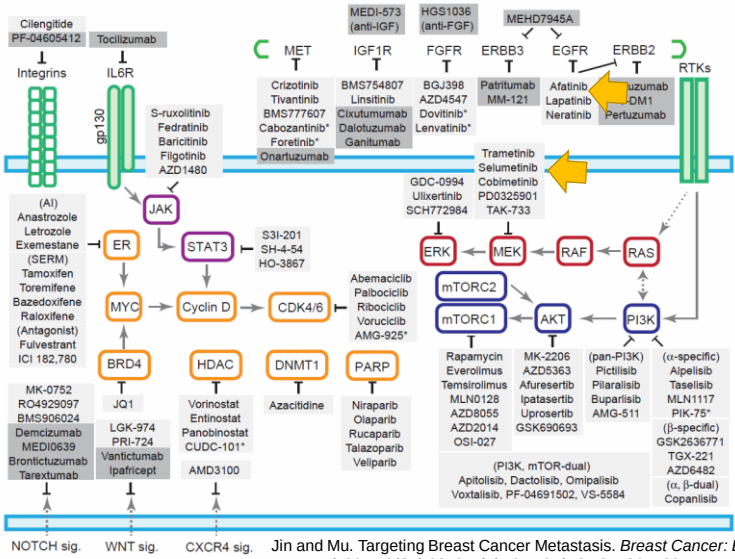

Standardized Whole-Blood Transcriptional Profiling Enables the Deconvolution of Complex Induced Immune Responses

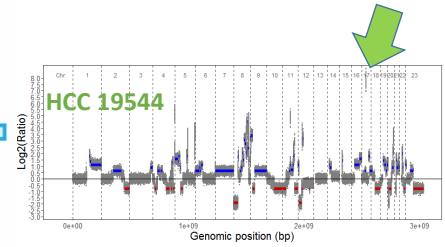
Cell Reports, 2016

Pipelines applied for precision analysis



What are the effects of treatments on HCC1954?



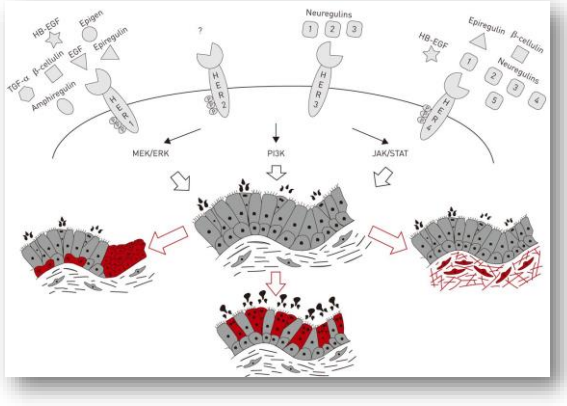


✓ PAM50 classification: Her2+

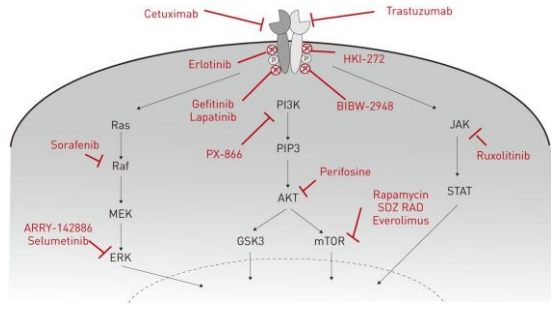
Jin and Mu. Targeting Breast Cancer Metastasis. *Breast Cancer: Basic and Clinical Research* 2015:9(S1) 23–34 doi:10.4137/BCBCR.S25460.

63

A simplified view of the HER family receptor family epidermal growth factor receptor (EGFR) signalling pathway in lung homeostasis and disease.

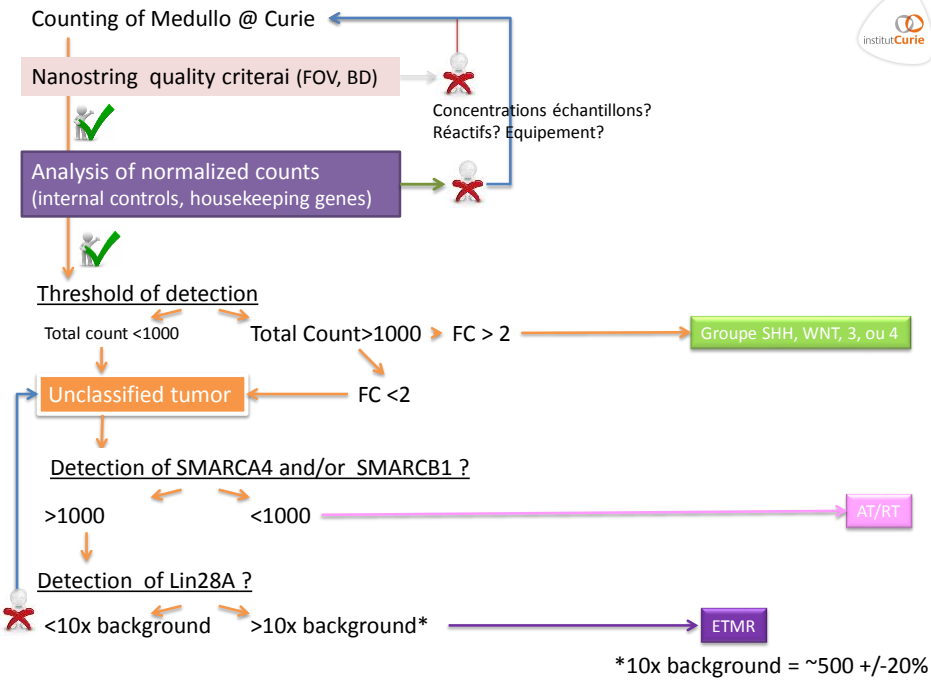


Clinically approved and in-development epidermal growth factor receptor (EGFR) signalling pathway inhibitors for chronic respiratory disease

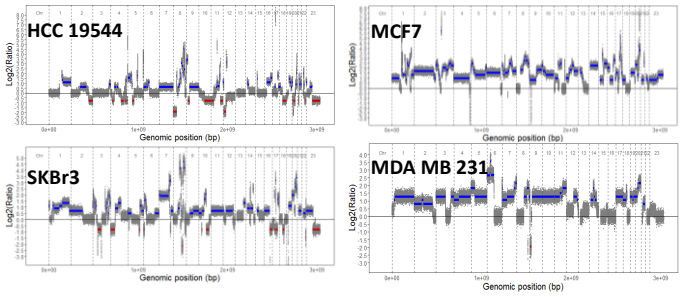


Sabari Vallath et al. Eur Respir J 2014;44:513-522

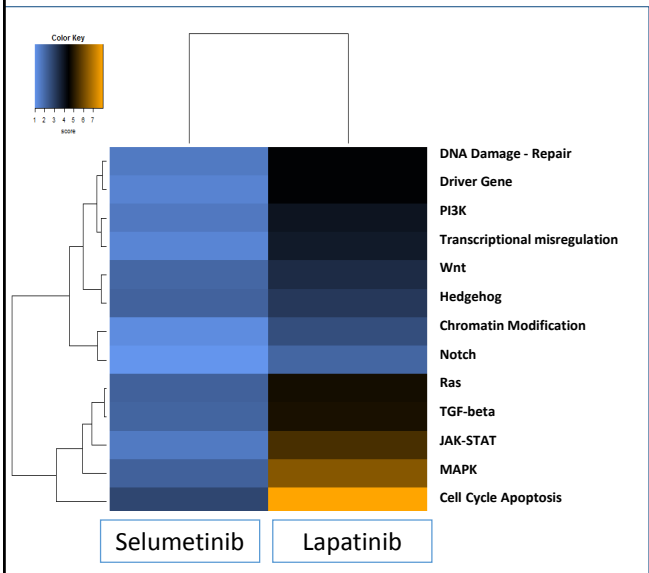
64



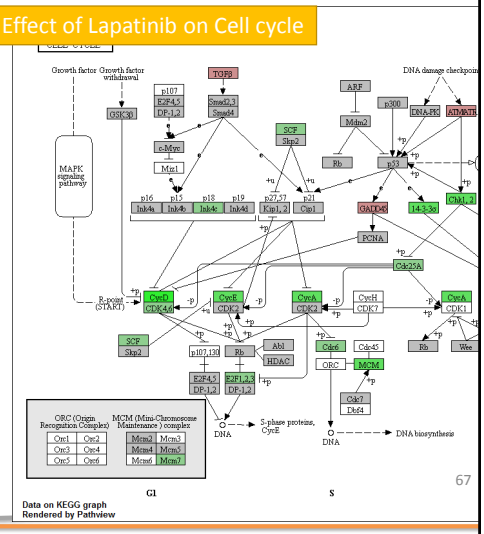
Whole Genome Copy Number analysis (Curie, Affymetrix SNP arrays)



Effect of Selumetinib and Lapatinib on HCC1954: Summary of a firts rapid Pathway analysis

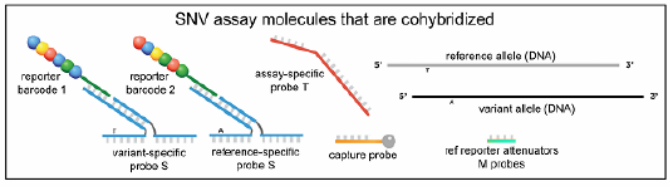


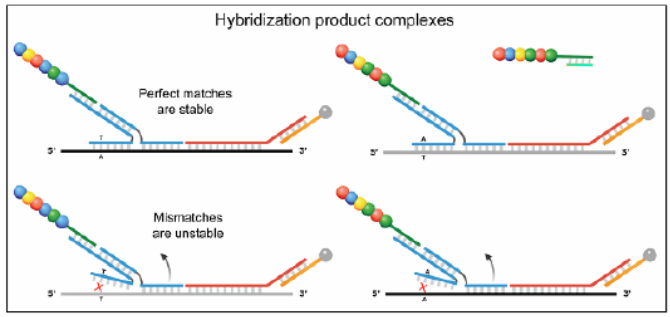
Effect of Lapatinib on Cell cycle



67

Vantage 3D™ DNA SNV Solid Tumor Panel for SNP detection

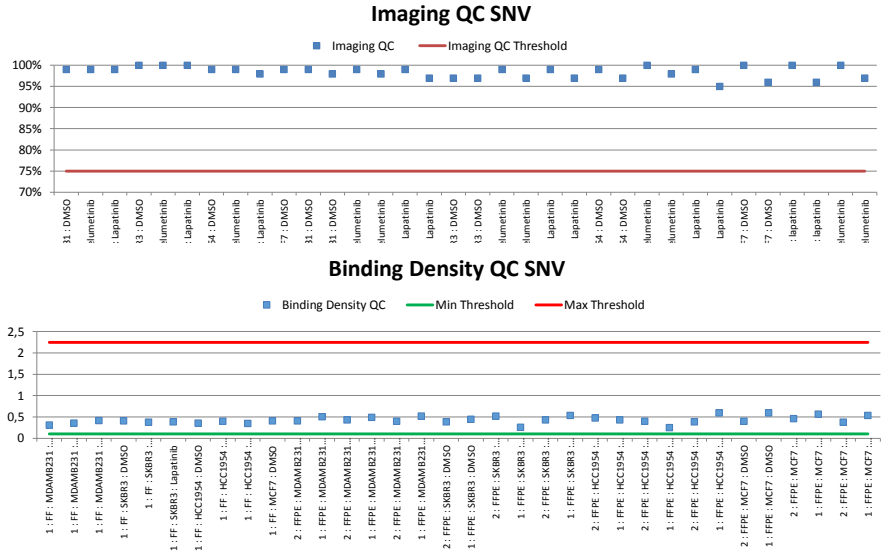




Product	Description	Catalog Number	Unit Size
nCounter® Vantage 3D DNA SNV Solid Tumor Panel (CSO)	Contains code set for 104 solid tumor actionable mutation over 25 genes. Includes Pre-Amplification Reagents. To be used as a stand-alone DNA assay or with nCounter Vantage 3D Protein and RNA. No Master Kit	VDXC-HST-12	12 Reactions
nCounter® Vantage 3D SNV Qualification Kit (CSO)	Contains synthetic oligos to qualify nCounter instruments before testing SNV Panel. To be used only one time for first time SNV users. No Master Kit.	VDXC-QualK-12	12 Reactions

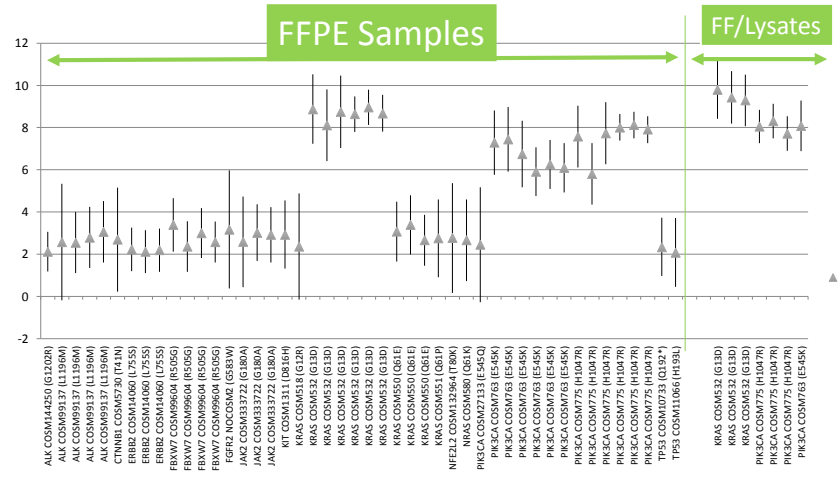
68

First quality controls of SNV experiments



Technical steps are validated

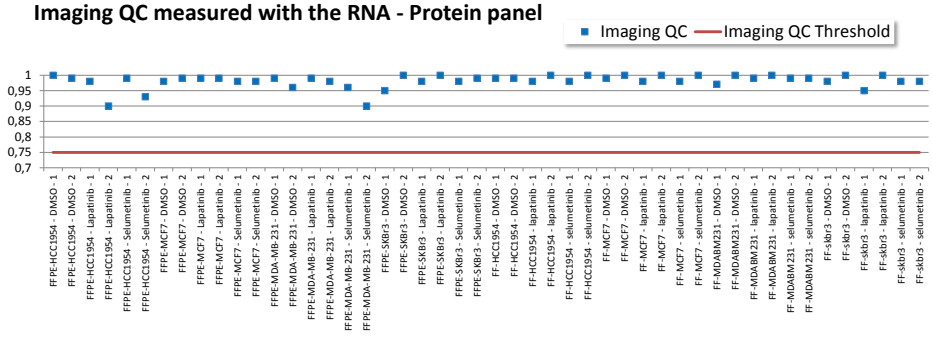
Log2 Fold Change is noised in FFPE samples



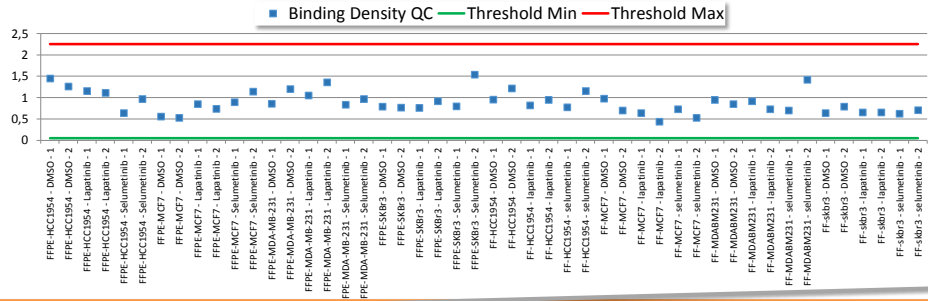
Breast cancer cell lines from lysates, show a correct mutational pattern, whereas BCCL from FFPE have a high rate of false positive for different genes.

Threshold of log2 FC need to be adjusted to distinguish False positive and true positive

Imaging QC measured with the RNA - Protein panel



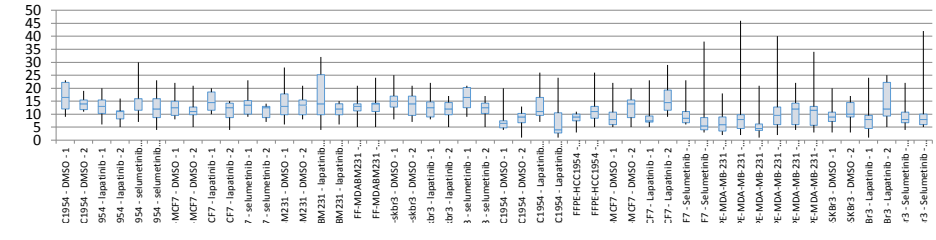
Binding density QC measured with the RNA - Protein panel



Technical steps are validated

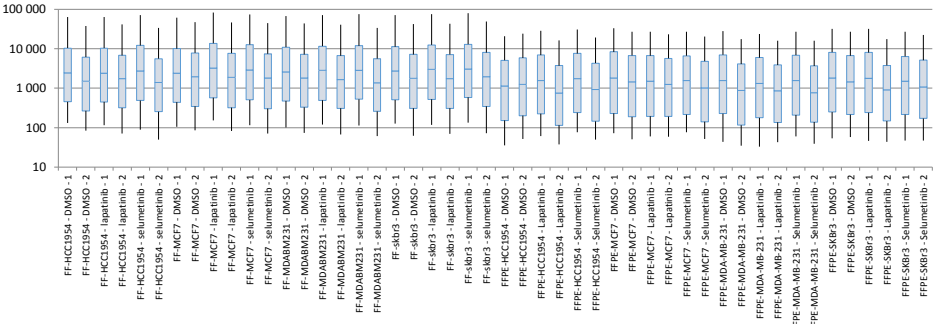
71

Background measured with the RNA - Protein panel: Negative controls



Negative control Mediane: 12
Max. Negative control: "F"=46

Positive controls

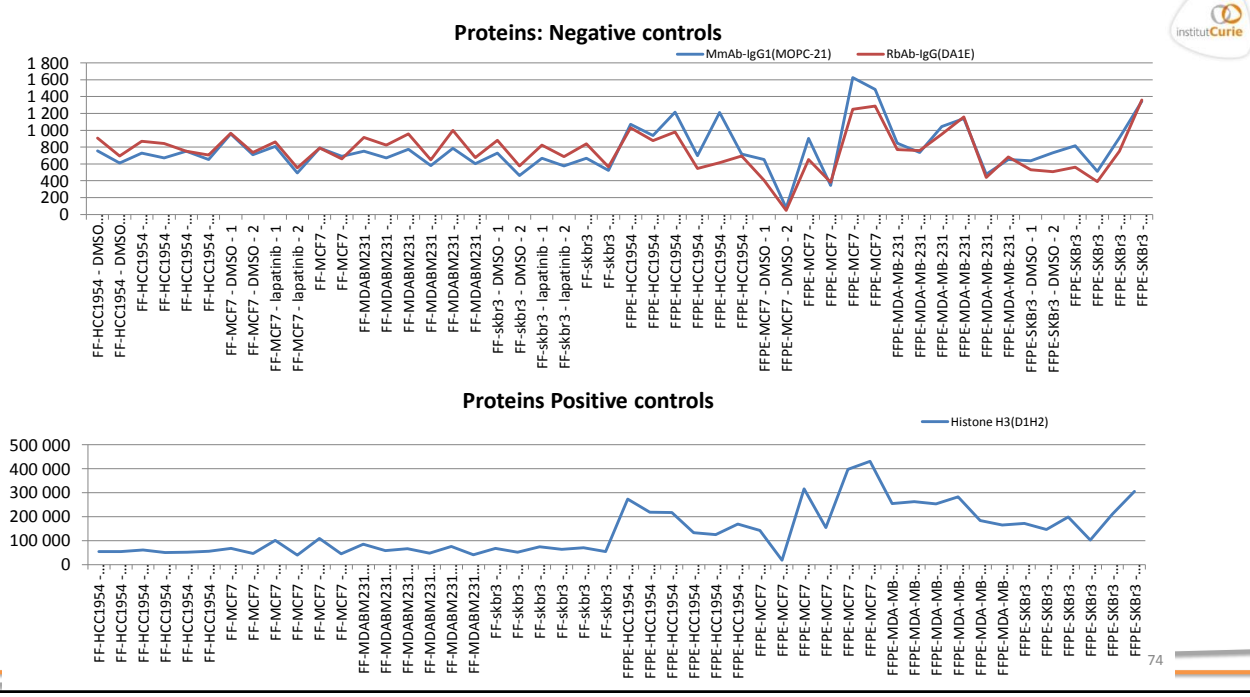


Spike in controls are homogenous

72

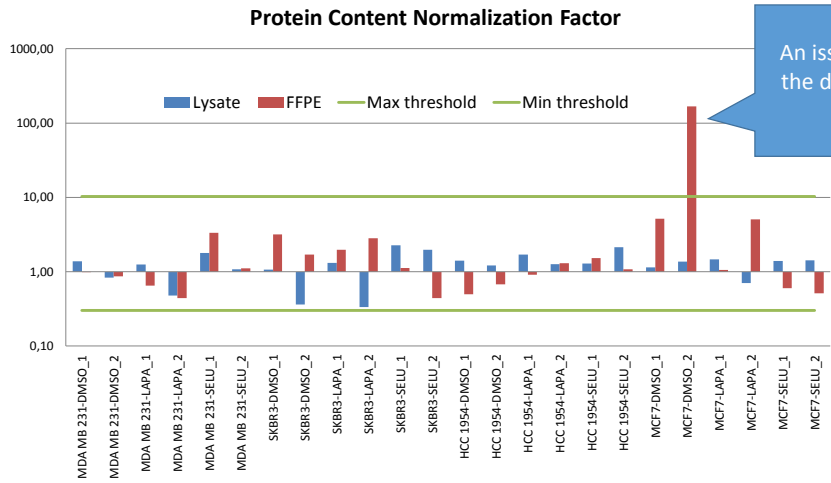
nCounter® Vantage 3D™ Protein Assay Normalization

73



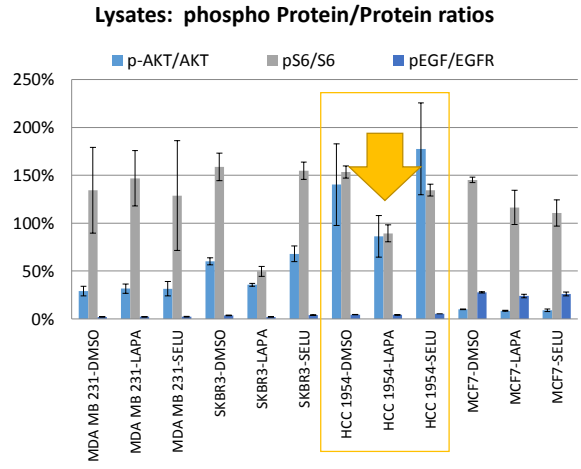
74

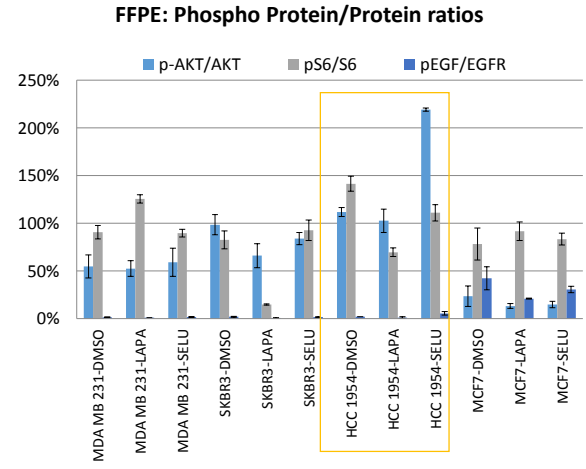
Normalization of Proteins data :
All protein geometric mean normalized counts



An issue was encountered during the delimitation of fixed cellswith the DakoPen.

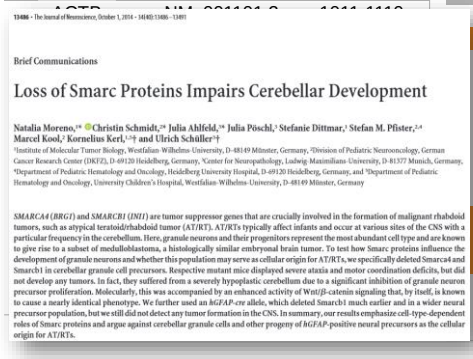
Comparisons of 3D Biology PanCancerPathway Panel :
Phospho Proteins vs Proteins





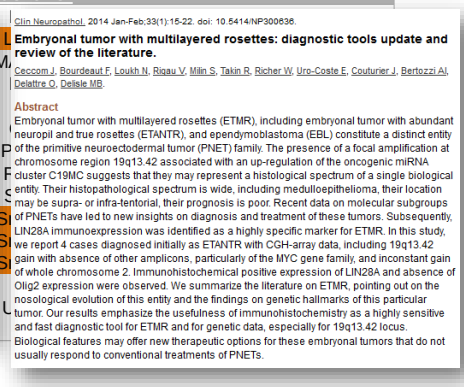
Customer Identifier	Accession	Targ Region

Customer Identifier	Accession	Targ Region



Brief Communications

Loss of Smarc Proteins Impairs Cerebellar Development



Clin Neuropathol. 2014 Jan-Feb;33(1):15-22. doi: 10.5414/NP300836.

Embryonal tumor with multilayered rosettes: diagnostic tools update and review of the literature.

Ceccom J, Bourdeaut F, Loukh N, Rigau V, Milin S, Takin R, Richer W, Uro-Coste E, Couturier J, Bertozzi AI, Delattre O, Delisle MB.

Abstract

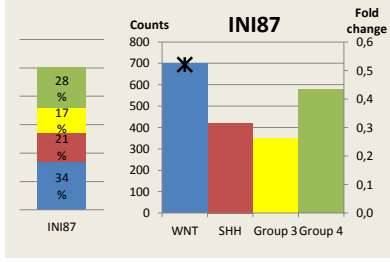
Embryonal tumor with multilayered rosettes (ETMR), including embryonal tumor with abundant neuropil and true rosettes (ETANTR), and ependymoblastoma (EBL) constitute a distinct entity of the primitive neuroectodermal tumor (PNET) family. The presence of a focal amplification at chromosome region 19q13.42 associated with an up-regulation of the oncogenic miRNA cluster C19MC suggests that they may represent a histological spectrum of a single biological entity. Their histopathological spectrum is wide, including medulloepithelioma, their location may be supra- or infra-tentorial, their prognosis is poor. Recent data on molecular subgroups of PNETs have led to new insights on diagnosis and treatment of these tumors. Subsequently, LIN28A immunoexpression was identified as a highly specific marker for ETMR. In this study, we report 4 cases diagnosed initially as ETANTR with CGH-array data, including 19q13.42 gain with absence of other amplicons, particularly of the MYC gene family, and inconstant gain of whole chromosome 2. Immunohistochemical positive expression of LIN28A and absence of Olig2 expression were observed. We summarize the literature on ETMR, pointing out on the nosological evolution of this entity and the findings on genetic hallmarks of this particular tumor. Our results emphasize the usefulness of immunohistochemistry as a highly sensitive and fast diagnostic tool for ETMR and for genetic data, especially for 19q13.42 locus. Biological features may offer new therapeutic options for these embryonal tumors that do not usually respond to conventional treatments of PNETs.

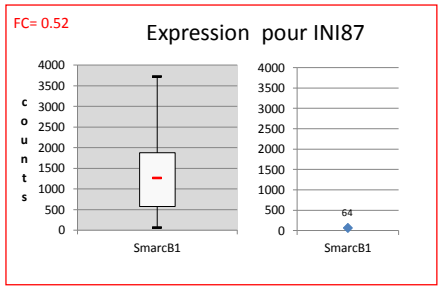
Added sequences for:

SMARCB1
SMARCA4 } **Atypical teratoid/rhabdoid tumours (AT/RTs)**

LIN28A > **Embryonal tumor with multilayered rosettes (ETMR)**

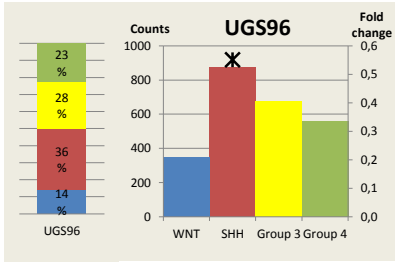
Utility of additional sequences to help ATRT and ETMR identification



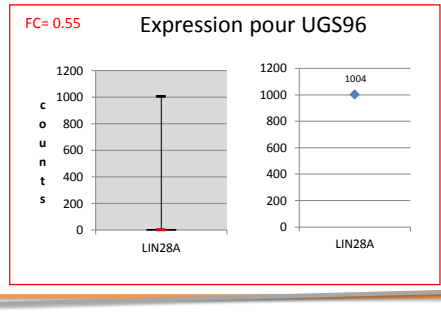


The weak expression of SMARCB1 / SMARCA4 suggest an ATRT

Utility of additional sequences to help ATRT and ETMR identification



High expression of LIN28A suggest an ETMR



Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013

Intrinsic subtype	Clinico-pathologic surrogate definition	Notes	Type of therapy
Luminal A	**'Luminal A-like'** *all of:* ER and PgR positive HER2 negative Ki-67 'low'[a] Recurrence risk 'low' based on multi-gene-expression assay (if available)[b]	The cut-point between 'high' and 'low' values for Ki-67 varies between laboratories.[a] A level of <14% best correlated with the gene-expression definition of Luminal A based on the results in a single reference laboratory. Similarly, the added value of PgR in distinguishing between 'Luminal A-like' and 'Luminal B-like' subtypes derives from the work of Prat et al. which used a PgR cut-point of ≥20%	Endocrine therapy is the most critical intervention and is often used alone.
Luminal B	**'Luminal B-like (HER2 negative)'** ER positive HER2 negative and *at least one of:* Ki-67 'high' PgR 'negative or low' Recurrence risk 'high' based on multi-gene-expression assay (if available)[b]	'Luminal B-like' disease comprises those luminal cases which lack the characteristics noted above for 'Luminal A-like' disease. Thus, either a high Ki-67[a] value or a low PgR value (see above) may be used to distinguish between 'Luminal A-like' and 'Luminal B-like (HER2 negative)'.	Endocrine therapy for all patients, cytotoxic therapy for most.
	'Luminal B-like (HER2 positive)' ER positive HER2 over-expressed or amplified Any Ki-67 Any PgR		Cytotoxics + anti-HER2 + endocrine therapy

[a]A majority of the Panel voted that a threshold of ≥20% was indicative of 'high' Ki-67 status. Others, concerned about the high degree of inter-laboratory variation in Ki-67 measurement and the possibility for undertreatment of patients with luminal disease who might benefit from chemotherapy, would use a lower (local laboratory specific) cut-point to define Ki-67 'high' or use multi-gene-expression assay results, if available.
[b]This factor was added during Panel deliberations after circulation of the first draft of the manuscript, to reflect a strong minority view. Although neither the 21-gene RS nor the 70-gene signature was designed to define intrinsic subtypes, a concordance study noted that over 90% of cases with a low RS and almost 80% of those with a 70-gene low-risk signature were classified as Luminal A.

Goldhirsch et al. Ann Oncol. 2013 September; 24(9): 2206–2223.

80