Molecular diagnostic tests to augment cytomorphologic diagnosis of Lung Cancer Trans-Thoracic Fine Needle Aspirate Samples

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Abstract

Morphologic analysis of cytology samples obtained by transthoracic needle aspiration (TTNA) is one of the primary methods for diagnosing bronchogenic carcinoma. However, across multiple studies the false negative rate for cytomorphological analysis ranges from 0.2-0.3, and among those diagnosed with lung cancer, the false positive rate for diagnosing the sub-class of small cell carcinoma averages 0.09. These false results lead to additional invasive diagnostic studies and delay treatment. In an effort to augment accuracy and utility of cytopathologic evaluation of TTNA lung samples, we measured the previously reported c-myc x E2F-1/p21WAF1/CIP1 (p21 hereafter) gene expression index in cDNA samples from 78 non-malignant lung tissues and 57 lung cancer tissues. Using the optimal cut-off value, this test correctly classified 72/78 non-malignant and 50/57 malignant samples for a correct classification rate of 90% (95% CI 83.6% - 94.3%), sensitivity of 88%, and specificity of 92%. A CDKN2C/FOSL1 test for distinguishing non-small cell lung cancer (NSCLC) from small cell lung cancer (SCLC) had a PPV of 100% with 63% sensitivity. In order to optimize the robustness and utility of these tests, we developed qPCR methods that enable simultaneous measurement of each target gene and reference gene transcript relative to a known number of internal standard competitive template (CT) molecules using two-color fluorometric analysis on real-time platform. For each gene, the native template was quantified with a sequence-specific FAM-labeled probe and the CT was quantified with a sequence-specific Quasar670 labeled probe. Results for each gene thus far demonstrate excellent linearity ($R^2 > 0.99$, slope 1.0 +/- 0.05), relative accuracy (variance < 0.2), signal-to-analyte response (1.0 +/- 0.05) and precision (CV < 30%) over six orders of magnitude, and reliable detection of as few as 10 molecules. Assessing both the c-myc x E2F-1/p21 index and the CDKN2C/FOSL1 test promises to augment cytomorphologic diagnosis of bronchogenic carcinoma in TTNA biopsy samples.

Introduction 1

NEED THAT IS BEING ADDRESSED:

❑ Tests that increase sensitivity and/or specificity of cytomorphologic methods in analysis of FNA lung samples.
❑ Tests that are robust: work reliably on small samples yielding degraded RNA, such as FNA samples, and cell-block prep of FNA samples.
❑ Specific needs:
1.If diagnoses of suspicious, atypical, and non-diagnostic are included in analysis as negative, cytomorphologic analysis false negative range is 0.2-0.3.
- Thus a test is needed that increases accuracy in diagnosing malignant vs non-malignant causes of chest lesions evident on imaging

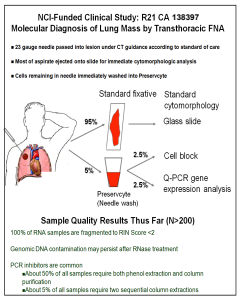
2.False positive cytomorphologic diagnosis for SCLC increases likelihood that someone will not be offered potentially curative surgery.
- Thus a test is needed that increases specificity (reduces false positive) in diagnosis of Small Cell Lung Cancer.

Method 1

LUNG CANCER DIAGNOSTIC TESTS
LCDT AND LCDT-SC

❑ Trans-thoracic FNA Sample available for Research Diagnostic Test Development

- Part of sample set aside in RNA preservative pending sign-out.
  - Best quality sample but interrupts standard workflow
  - We are validating LCDT and LCDT-SC in new set of such samples in current study.
- Sections from cell block.
  - Lower quality sample but does not interrupt standard workflow
  - We are assessing LCDT and LCDT-SC in these samples in current study.



Results 1

LUNG CANCER DIAGNOSTIC TESTS

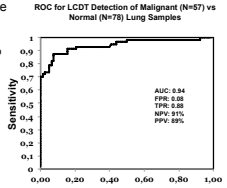
TEST	Clinical Validation Results					Prospective Clinical Validation Trial
	Subjects (N)	Accuracy (%)	Sensitivity (%)	Specificity (%)	ROC AUC (%)	
LCDT test to augment cytomorphology in diagnosis of lung cancer[2]	135	90	88	92	0.94	Currently underway
LCDT NSCLC/SCLC Classifier test to augment cytomorphology in discriminating between NSCLC and SCLC[3]	65	95	63	100	0.84	Currently underway

[1]Blomquist et al, Cancer Research, 2009; [2]Manuscript in preparation, Willey et al; [3]Manuscript in preparation, Willey et al

LCDT TEST TO INCREASE ACCURACY OF LUNG CANCER DIAGNOSIS IN CYTOLOGY SAMPLES

❑ Cytomorphologic False Negative Rate (FNR) for analysis of trans-thoracic needle aspiration samples: 0.20-0.30
- E.g. Zarbo and Preiser, Arch Pathol Lab Med 1992; 116:463–470

❑ LCDT FNR: 0.09

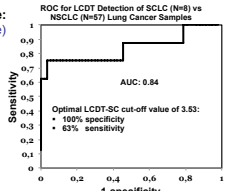


Conclusion:
❑ LCDT will augment cytomorphologic analysis to reduce need for repeat invasive tests

LCDT-SC TEST TO INCREASE SPECIFICITY OF DIAGNOSING SCLC IN CYTOLOGY SAMPLES

❑ **Cytomorphology specificity alone:** 91% (range 0.0-33% from literature)
- Knudsen et al. Acta Radiol 1996; 37:327–331 (25%): Payne et al. J Clin Pathol 1981; 34:773–778 (33%)

❑ **LCDT-SC specificity alone:**
- 100%



Conclusions:
❑ LCDT-SC will augment cytomorphologic analysis

❑ High specificity will minimize likelihood that a patient will erroneously be diagnosed as SCLC and therefore be denied potentially curative surgery.

Introduction 2

OPPORTUNITIES AND PROBLEMS

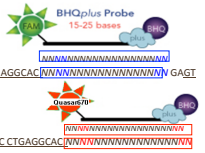
❑ **Opportunity:** Large archives of formalin-fixed paraffin-embedded (FFPE) samples from subjects with known response to specific treatment.

❑ **Opportunity:** FNA (Fine Needle Aspirate) is the most common method for diagnosing advanced lung cancer.
- These samples are key to development of biomarkers that predict response to chemotherapy ("Personalized Medicine").

❑ **Problem:** RNA quality in these samples is variable and typically poor
- RT-PCR methods that measure reliably in FFPE and FNA samples must be developed and validated.

Method 2

➢Select primers
➢Test Primers for Efficiency
➢Synthesize NT, CT, NT specific probe, CT specific probe
➢Test Probes for Specificity.



❑ NT has four to six bases different compared with CT.
❑ NT and CT probe each binds specifically and then emits different color.

Results 2.1

PROBE SPECIFICITY:
-Test binding to non-homologous strand

Example: ACTB NT Probe Specificity Test

ACTB NT or CT	NT probe + NT Ct	NT probe+ CT Ct	Expected value	Measured CT value	Measured CT/ Expected CT)%
[10^-11]M	16.7	34.0	6000000	37	0.00
[10^-12]M	20.3	36.0	600000	12	0.00
[10^-13]M	23.8	None	60000	0	0.00
[10^-14]M	27.2	None	6000	0	0.00
[10^-15]M	30.3	None	600	0	0.00
[10^-16]M	34.0	None	60	0	0.00

Specificity Results

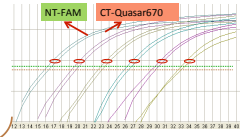
	NT probe(%)	CT probe(%)
ACTB	99.99	99.2
cMyc	100	99.8
p21	100	100
E2F1	99.86	100

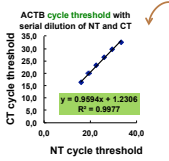
❑ Result: Probe binding was highly specific (<1% binding to the non-homologous strand) at the cycle threshold for all four genes.

AMPLIFICATION PLOT

1:1 NT/CT

ACTB(1/1) [NT+CT]	NT Ct	CT Ct	Delta Ct
[10^-11]+[10^-11]M	16.0	16.4	-0.32
1/10	19.4	19.9	-0.51
1/100	22.8	23.1	-0.38
1/100	26.1	26.5	-0.37
1/1000	29.5	29.9	-0.39
1/10000	33.2	32.7	0.56
Mean			-0.24



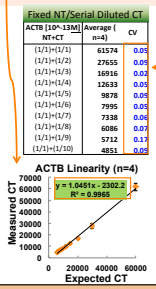


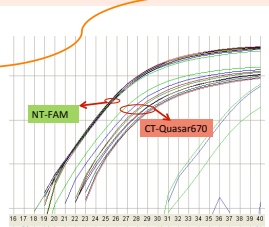
Key points: As NT/CT mixture is diluted, the variation in delta Ct is low.
❑ This method is linear over full range of PCR.
❑ Difference in NT vs CT PCR product fluorescence may vary due to: difference in concentration measurement, difference in efficiency of probe label and/or fluor specific activity.
❑ NT/CT mixture as inter-experimental reference material for inter-experimental controls for these sources of variation.

ABILITY TO DETECT SMALL DIFFERENCE BY CYCLE THRESHOLD
-Linearity (Slope & Correlation Coefficient(R^2)) and Precision(CV)

Fixed NT/Serial Diluted CT

ACTB [10^-13M] NT+CT	Average (n=4)	CV
(1/1)-(1/1)	61574	0.05
(1/1)-(1/2)	27655	0.05
(1/1)-(1/3)	16916	0.03
(1/1)-(1/4)	12633	0.05
(1/1)-(1/5)	9878	0.05
(1/1)-(1/6)	7995	0.05
(1/1)-(1/7)	7338	0.06
(1/1)-(1/8)	6086	0.07
(1/1)-(1/9)	5712	0.17
(1/1)-(1/10)	4851	0.05



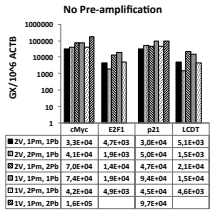


❑ Good linearity (S=1.045, R^2=0.997) and Precision (CV (<0.2)).

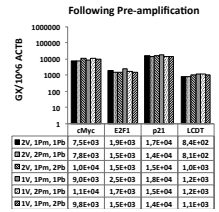
Results 2.2

COMPARISON OF PRE-AMP AND NO PRE-AMP:
-Test with different reaction volumes, primer and probe concentrations

Effect of Different PCR Conditions on Analysis of cDNA from Normal Lung RNA



	cMyc	E2F1	p21	LCDT
2V, 1Pm, 1Pb	3,3E+04	4,7E+03	3,0E+04	5,1E+03
2V, 2Pm, 1Pb	4,1E+04	1,9E+03	5,0E+04	1,5E+03
2V, 1Pm, 2Pb	7,0E+04	1,4E+04	4,7E+04	2,1E+04
1V, 1Pm, 1Pb	7,4E+04	1,9E+04	9,4E+04	1,5E+04
1V, 2Pm, 1Pb	4,2E+04	4,9E+03	4,5E+04	4,6E+03
1V, 1Pm, 2Pb	1,6E+05		9,7E+04	

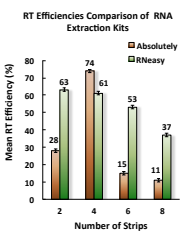


	cMyc	E2F1	p21	LCDT
2V, 1Pm, 1Pb	7,5E+03	1,9E+03	1,7E+04	8,4E+02
2V, 2Pm, 1Pb	7,8E+03	1,5E+03	1,4E+04	8,1E+02
2V, 1Pm, 2Pb	1,0E+04	1,5E+03	1,5E+04	1,0E+03
1V, 1Pm, 1Pb	9,0E+03	2,5E+03	1,8E+04	1,2E+03
1V, 2Pm, 1Pb	1,1E+04	1,7E+03	1,5E+04	1,2E+03
1V, 1Pm, 2Pb	9,8E+03	1,5E+03	1,4E+04	1,1E+03

❑ Less variation after 20 cycles of pre-amplification with mixture of primers for four genes (2nd round amplification performed 1/1000 dilution of pre-amplified product). Lower variation might result from higher signal to background following pre-amplification.

COMPARISON OF TWO EXTRACTION KITS

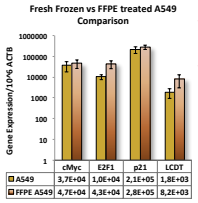
Experimental samples:				
# strips	2	4	6	8
# wells/strip	8	16	24	32
# microns (µm)	40	80	120	160
surface area (mm²)	1152	2304	3456	4608



	Absolutely				RNeasy			
	A2	A4	A6	A8	R2	R4	R6	R8
Con. (µg/µl)	0.016	0.082	0.056	0.031	0.056	0.039	0.067	0.009
260/280A	2.06	2.02	1.98	1.96	2.05	2.07	2.13	3.65
RT effic. (%)	27.6	73.9	15.3	10.7	63.1	60.8	53.1	37.4
RIN Score	1	2.1	1.2	1	1.9	1.3	2.1	1
Total (µg)	0.48	2.45	1.69	0.94	1.68	1.17	2.01	0.26

❑ In general, RNeasy® and Absolutely® produced similar yields and purities.
❑ RNA extracted with RNeasy® had a statistically significantly better RT efficiency, and this kit took less time. See abstract #5538 for RT efficiency measurement method.

FRESH FROZEN vs FFPE COMPARISON WITH A549



	cMyc	E2F1	p21	LCDT
A549	3,7E+04	1,0E+04	2,1E+05	1,8E+03
FFPE A549	4,7E+04	4,3E+04	2,8E+05	8,2E+03

❑ LCDT of FFPE treated A549 was higher than fresh frozen but showed similar results.
❑ Pre-amplification showed less variation than no pre-amplification.

❑ Measured LCDT with pre-amplification:

	LCDT
Normal Lung Tissue RNA	1100
A549(NSCLC)	1800
H23 (NSCLC)	11000
H146 (SCLC)	72000
#2 H. Malignant FNA FFPE block	84000
#3 H. Malignant FNA FFPE block	29000

Future Work

❑Test the feasibility of implementing LCDT test for FNA or surgically removed FFPE samples.
- Generate a cut-off value for lung cancer diagnosis of malignancy.
- First priority will be FFPE because these are samples that are generated in typical Pathology lab.

❑ Test the reproducibility, robustness in PCR-based molecular diagnostic analysis of FNA and FFPE samples.

Acknowledgement

❑ Test development supported by CA138397 and CA132806
❑ Tests licensed to Accugenomics, Inc., Wilmington, NC

Competing Interests

❑ J. Willey serves as consultant to and has significant interest in Accugenomics