Use of BHQplus™ Probes to Optimize Multiplex Competitive qRT-PCR in Diagnostics

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Outline

- **Competitive qRT-PCR**
  - Advantages in discovery and diagnostics
  - Applications in diagnostic tests for infectious microbes
- **Multiplex competitive qRT-PCR Test Design***
  - Multiplex pre-amplification with mixture of internal standards
  - Second round amplification with BHQplus probes
- **Applications in diagnostics**
  - Lung cancer diagnostic test (LCDT)
    - Diagnosis of Formalin Fixed Paraffin Embedded (FFPE) samples
  - Viral Hemorrhagic Septicemia (VHS) in fish test.

*Multiplex competitive qRT-PCR technology and the LCDT are protected by approved and/or pending patents licensed to Accugenomics, Inc.

Advantages of Competitive qRT-PCR in Diagnostics

- **PCR each target native template (NT) in competition with a known number of respective synthetic internal standard (IS) molecules.**
  - NT and IS compete for same primers
- **Experiment Set-up**
  - Mix each sample with a known concentration of IS
    - This fixes the concentration of transcript target native template (NT) in sample relative to its respective IS template.
  - Aliquot sample/IS mixture into each well containing PCR reagents and primers
  - Primers will amplify the NT and IS in competition.
- **Advantages**
  - Provides absolute quantification without need for external standard curve.
  - Controls for interfering substances and false negatives

Applications: Use of Synthetic Competitive Internal Standards In Diagnostics

- Examples in Commercial Diagnostics
  - FDA-approved tests for infectious microbes
    - e.g., Roche COBAS® Ampliscreen HIV-1 Test
  - A single analyte (HIV-1 gene) is measured relative to a known number of synthetic internal standard molecules

Applications: Use of Synthetic Competitive Internal Standards In Diagnostics

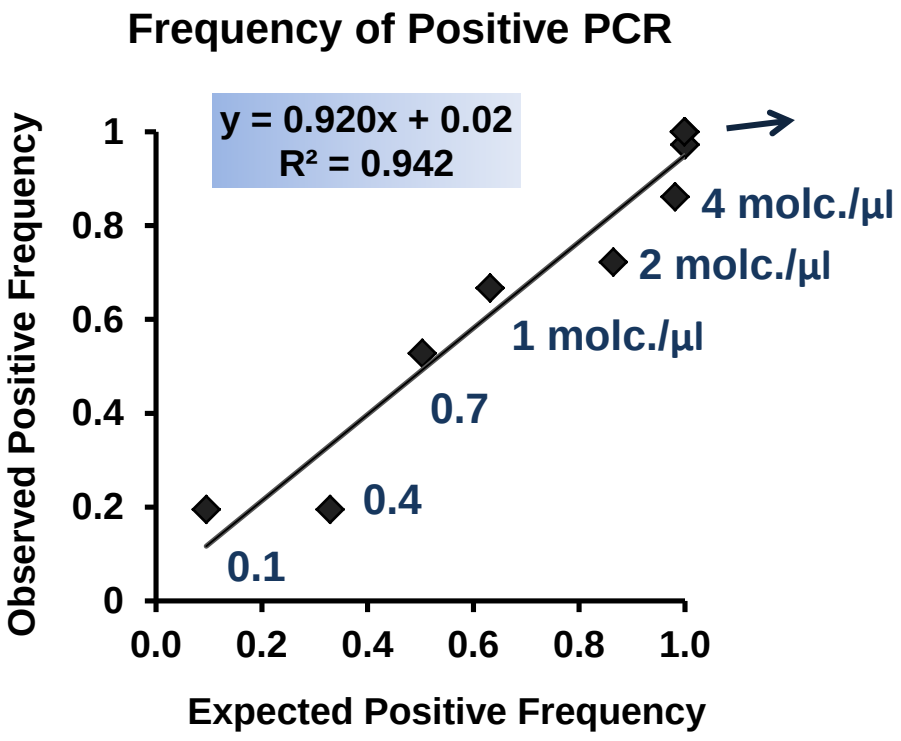
- ***Challenge:*** ***Many transcript abundance based tests require measurement of at least two analytes:***
  - *At least one reference gene and respective internal standard*
  - *At least one target gene and respective internal standard*
- **Solutions:**
  - **Use a mixture of synthetic internal standards (MIS)**
    - **Accugenomics, Inc. patents, e.g. U.S. No. 7,527,930)**
  - **Multiplex competitive qRT-PCR**
- ***Challenge:*** ***Most lung cancer biopsies are small and degraded***
  - *Fine needle aspirate provides very small samples*
  - *Formalin fixation and paraffin embedding (FFPE) for cytomorphologic evaluation* damages RNA and DNA, reduces RT and PCR efficiency 100-fold
- **Solutions:**
  - **1st round multiplex of sample with MIS to conserve sample/amplify signal**
  - **2nd round PCR with single primer pair and NT and IS BHQplus probes**

Outline of Test Development Approach

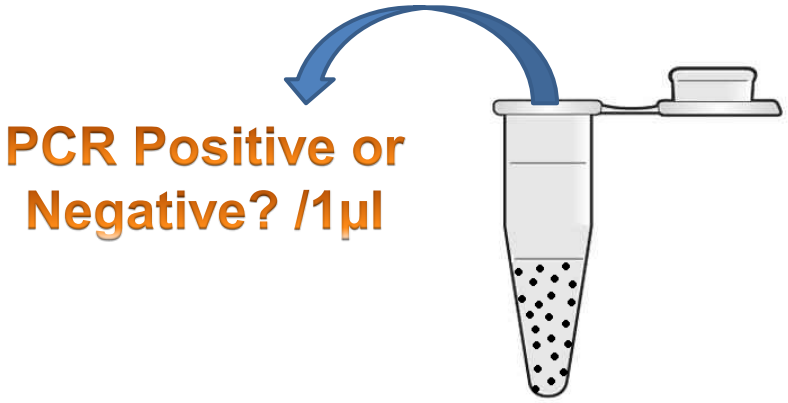
- **Develop standardized two color fluorometric assays** for
  - Four gene Lung Cancer Diagnostic Test (LCDT):
    - E2F1, MYC, CDKN1A, ACTB (Warner, Journal of Molecular Diagnostics, 5, 16-183, 2003; Yeo, PlosOne, 9, 2014)
    - Fish Viral Hemorrhagic Septicemia Virus (Pierce, PlosOne, 8, 2013)
- **Prepare reagents**
  - Synthetic internal standard (IS) for each gene
  - Mixture of Internal Standards (MIS).
  - FAM probe for each NT and Quasar670 probe for each IS
- **Conduct Analytical Validation Experiments**
- **Evaluate optimized assay in diagnostic samples**.

Digital PCR Quantification of Synthetic Internal Standards (60-80 bp)

Limiting Dilution



- Nine replicates at 10 dilution points (40, 20, 10, 7, 4, 2, 1, 0.7, 0.4, 0.1 molecules/µl).



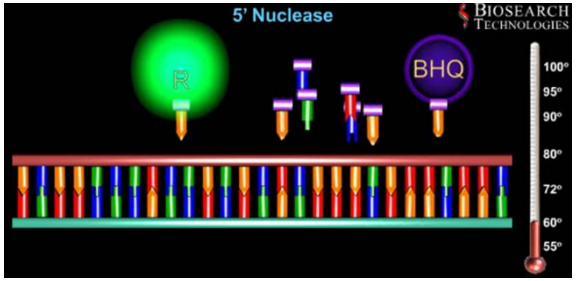
- ✓ **Internal standard molecule concentrations were accurate.**
- ✓ **Primers for each gene amplified a single molecule.**
- ✓ **Primers had 100% efficiency.**

TWO COLOR FLUOROMETRIC ASSAY Development

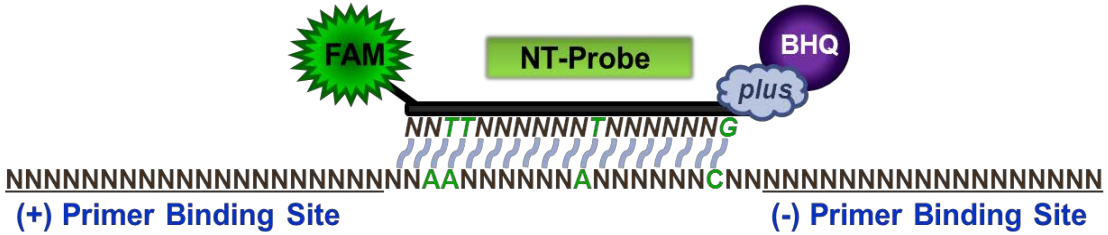
-SEQUENCE-SPECIFIC FAM PROBE for NT

-SEQUENCE-SPECIFIC QUASAR PROBE FOR INTERNAL STANDARD (IS)

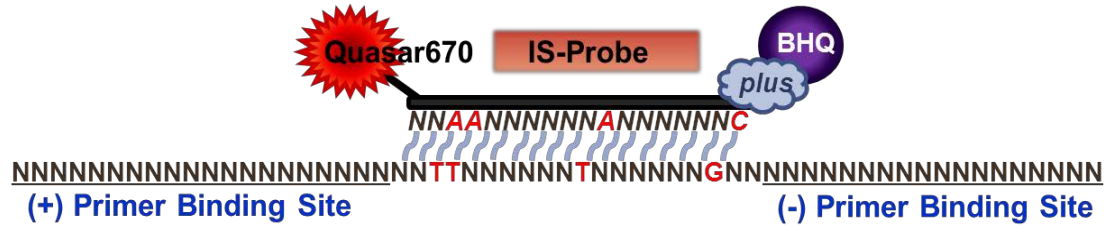
Example: ACTB
(60bp PCR product)

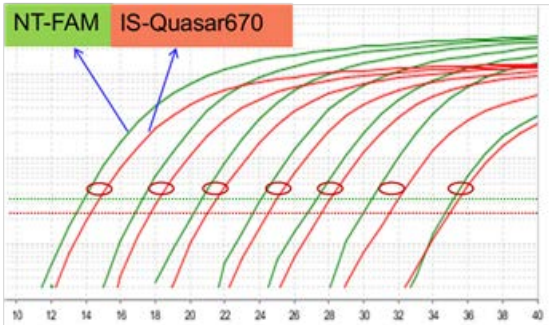


Native Template (NT):



Internal Standard (IS):





- IS designed with four to six bases different (red N) from NT (green N).
- NT probe homologous to NT sequence labeled with **FAM** fluor.
- IS probe homologous to IS sequence labeled with **Quasar670** fluor.
- NT and IS compete for same primers and amplify with same efficiency
- NT amount quantified as NT (FAM) threshold cycle (Ct)– IS (Quasar670) threshold cycle (Ct).

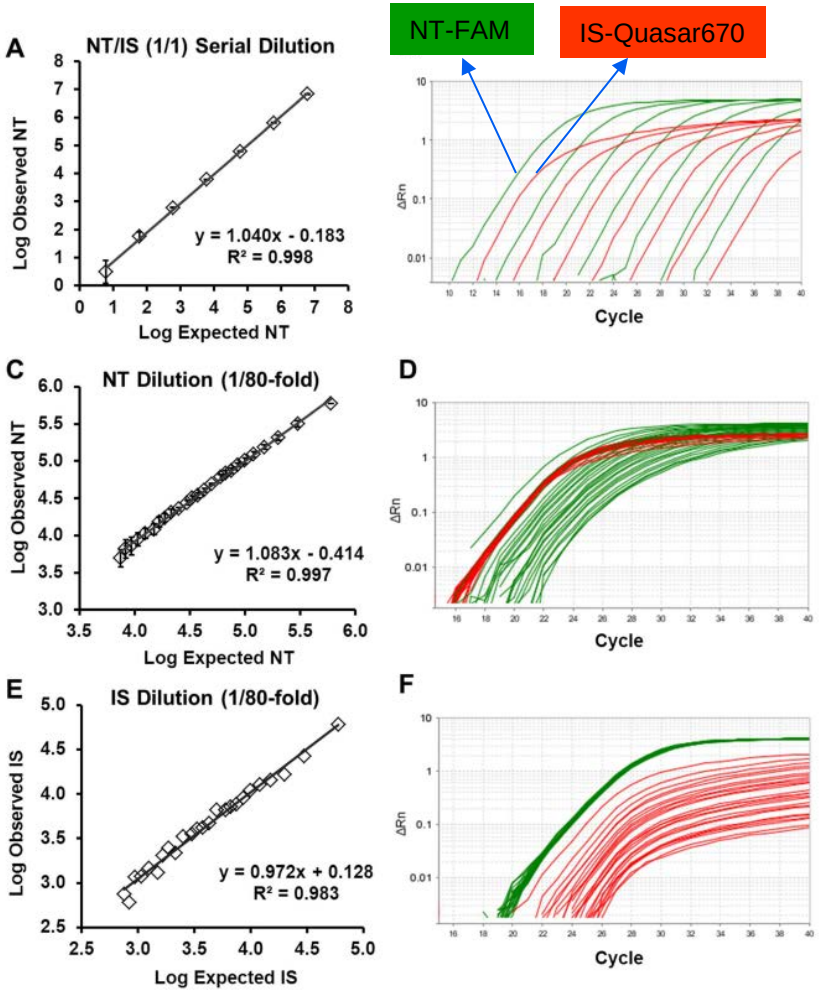
Analytical Validation: Linearity

Observed compared to expected E2F1values measured in dilution series samples by two-color fluorometric assay

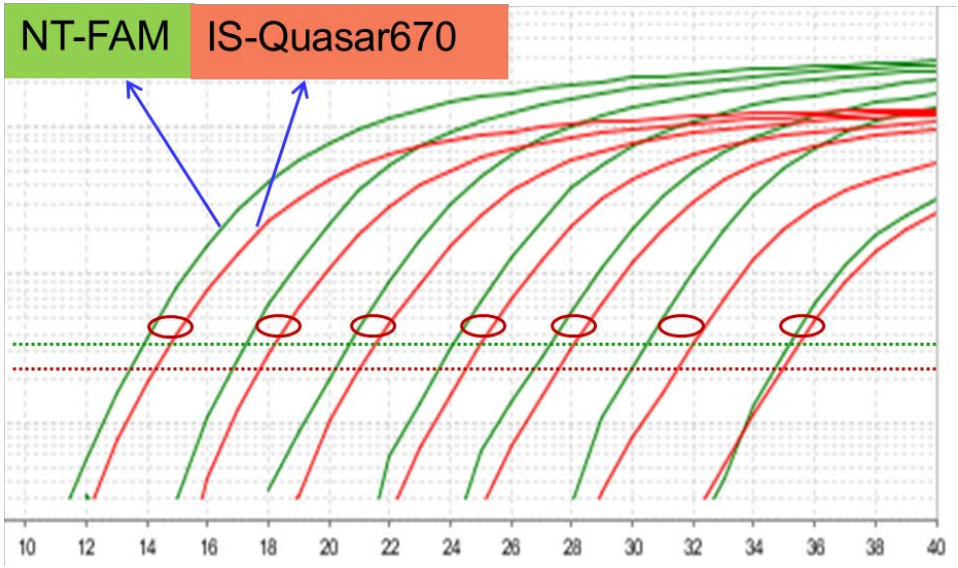
- **E2F1**
  - linearity graphs (A, C, E)
  - amplification plots of (B, D, F).
- **(A, B)**, dilution of external standards mixture (ESM, 1/1 mixture of NT/IS)
  - 10^{-11} M through 10^{-17} M
- **(C, D),** NT dilution relative to constant IS
  - from 1/1 NT/IS (10^{-12}M) to 1/80 (NT/IS)
- **(E, F),** IS dilution relative to constant NT
  - from 1/1 NT/IS (10^{-13}M) to 1/80 (IS/NT)

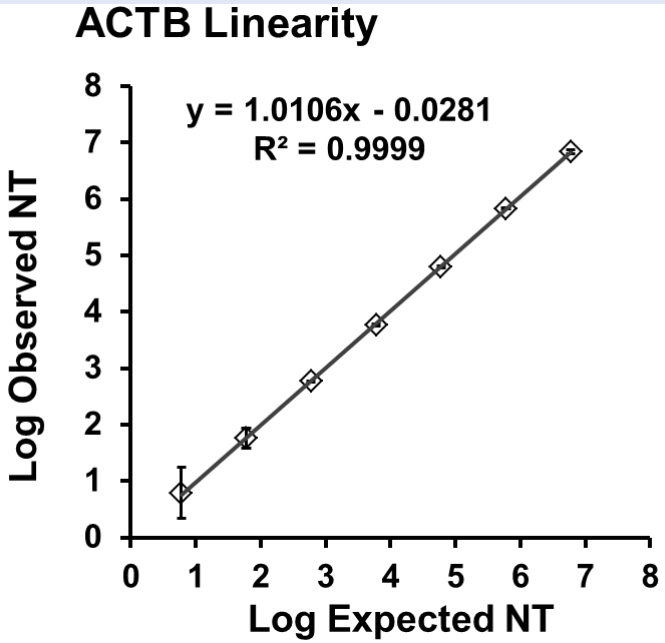
Summary

- **Measurement of sample relative to two internal standard mixtures covers nearly 8-logs$_{10}$**



Analytical Validation: Precision/Sensitivity





- **Low imprecision (CV < 10%) over five orders of magnitude and.**
- CV <30% down to <100 molecules
- Reliable detection of as few as 10 molecules.

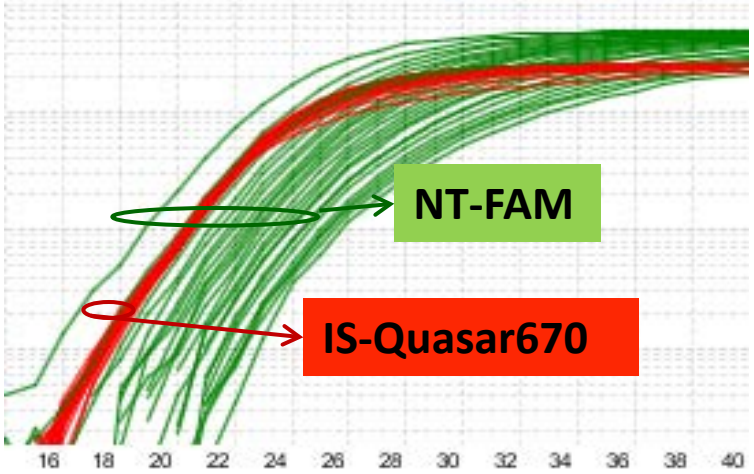
ACTB			
1:1 Dilution	Expected NT	Average	CV
10^{-11}M	6000000	6780000	0.09
10^{-12}M	600000	671000	0.02
10^{-13}M	60000	62600	0.05
10^{-14}M	6000	5880	0.04
10^{-15}M	600	577	0.04
10^{-16}M	60	61	0.42
10^{-17}M	6	9	1.06
Average of CV from 10^{-11}M to 10^{-16}M			**0.11**
Average of CV from 10^{-11}M to 10^{-17}M			**0.25**

Precision

-serially diluted NT relative to IS

NT Dilution	Expected NT	Average	CV
NT 1/1	600000	600000	0
NT 1/2	300000	317000	0.09
NT 1/3	200000	217000	0.16
NT 1/4	150000	156000	0.11
NT 1/5	120000	124000	0.10
NT 1/6	100000	99700	0.13
NT 1/7	85700	87400	0.15
NT 1/8	75000	71300	0.14
NT 1/9	66700	67400	0.14
NT 1/10	60000	61000	0.14
Average from 1/1 to 1/20 dilution			**0.12**
NT 1/12	50000	48000	0.19
NT 1/14	42900	37300	0.19
NT 1/16	37500	31900	0.16
NT 1/18	33300	30600	0.15
NT 1/20	30000	27400	0.19
Average from 1/1 to 1/20 dilution			**0.14**
NT 1/24	25000	22500	0.20
NT 1/28	21400	19100	0.22
NT 1/32	18800	16600	0.23
NT 1/36	16700	14600	0.31
NT 1/40	15000	12100	0.31
Average from 1/1 to 1/40 dilution			**0.17**
NT 1/48	12500	10200	0.25
NT 1/56	10700	8000	0.34
NT 1/64	9380	6800	0.34
NT 1/72	8330	5700	0.35
NT 1/80	7500	3600	0.45
Average from 1/1 to 1/80 dilution			**0.20**

- CV amount four replicates at each dilution was < 10%.
- As dilution increases, increasing CV and slight deviation of slope from 1.0



Calculation of Transcript Abundance

Quantify the copy number for each gene NT in a cDNA Sample

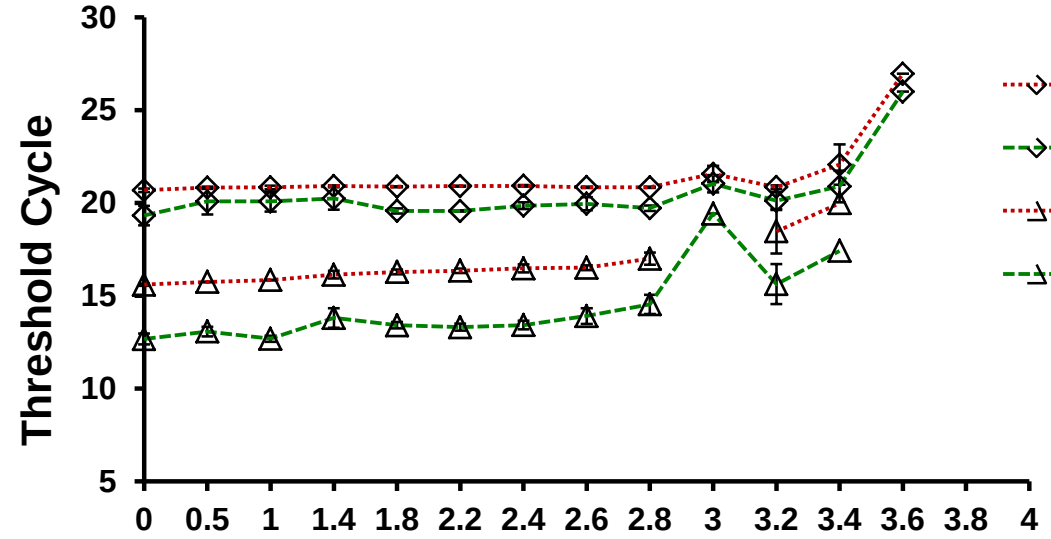
- Calculate [NT Cq - IS Cq] for the unknown sample
- Multiply $2^{(-\text{delta Cq})}$ x input IS copies in reaction = NT copy

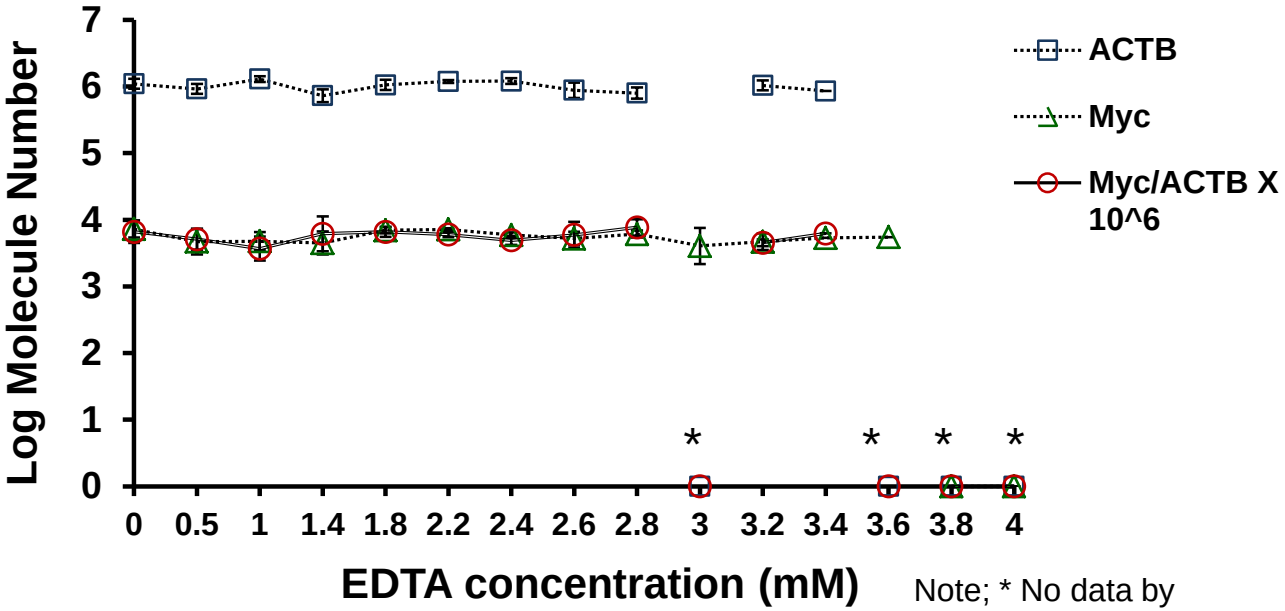
Normalize Target Gene to Loading Control Gene (e.g. ACTB)

- Each Target Gene NT value normalized to the ACTB loading control gene NT value
- Final value: Target Gene NT molecules/10^6 ACTB molecules

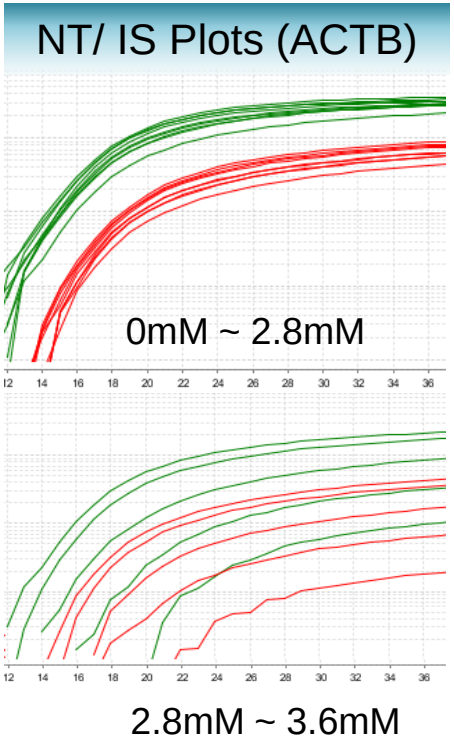
Analytical Validation: Robustness

Internal standards control for interfering substances (e.g. EDTA)





Note; * No data by No Ct results



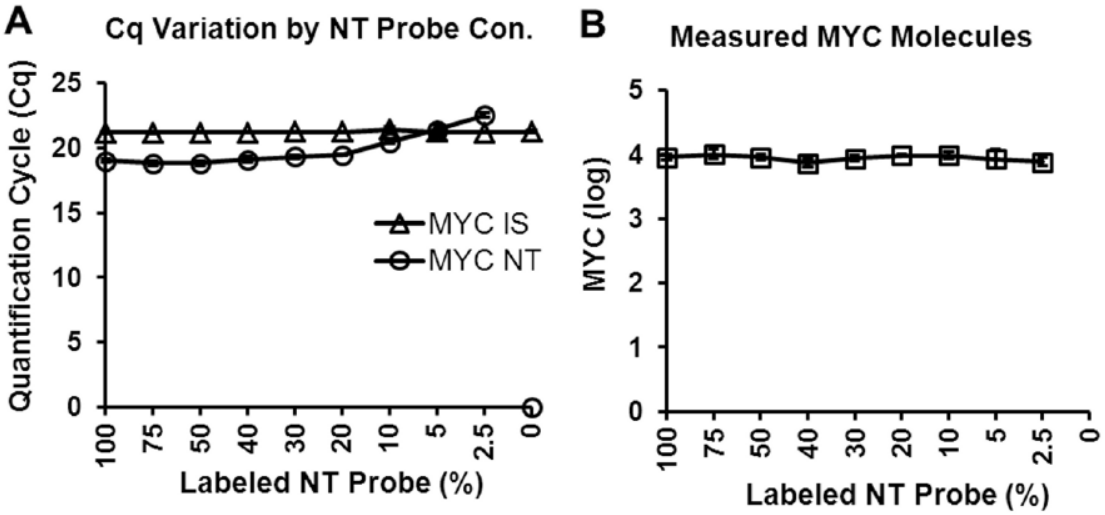
- False negative prevention
- Accurate GX measurements

Analytical Validation: Robustness

External Standards Mixture (ESM) Controls for Variation in Fluor signal or Cq Selection

- ESM contains known 1:1 concentration of each synthetic NT and IS
- Same ESM used in each experiment.
- ESM corrects for variation in experimental conditions, including:
  - Fluorescence specific activity (S.A.) (i.e., [labeled probe]/[total probe])
    - S.A. may vary between experiments due to freeze thaw, lot differences
  - Cq selection; software may be affected by experimental conditions.
- Sample [NT Cq – IS Cq] values corrected relative to ESM [NT Cq – IS Cq] values.

- A. Effect of diluting labeled probe with unlabeled probe on MYC measurement
- B. Correction with ESM controls for variation resulting from instability or intensity differences of two fluors.



Yeo et al, PlosOne, 9, 2014)

Correction for Variation in Fluor/Cq

Quantify the copy number for each gene NT in a cDNA Sample

- Calculate [NT Cq - IS Cq] for the unknown sample
- *Calculate average [NT Cq - IS Cq]ESM of two concentrations of ESM*
- *Calculate the corrected delta Cq as: [NT Cq - IS Cq]Sample - [NT Cq - IS Cq]ESM*
- Multiply $2^{(-\text{corrected delta Cq})}$ x input IS copies in reaction = NT copy

Normalize Target Gene to Loading Control Gene (e.g. ACTB)

- Each Target Gene NT value normalized to the ACTB loading control gene NT value

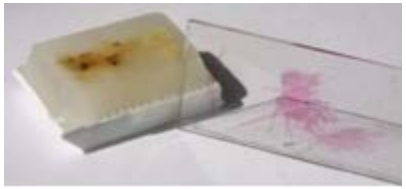
- Final value: Target Gene NT molecules/10^6 ACTB molecules

Summary of Test Development

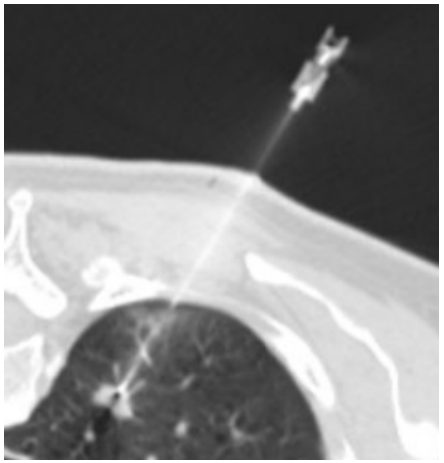
- Synthetic Internal Standard for each gene
  - Controls for interfering substances in PCR, prevents false negatives
- Mixture of Internal Standards (MIS) (Accugenomics, Inc.)
  - Controls for pipetting variation
- BHQplus probes: **FAM label** for each NT probe, **Quasar670** probe for each IS probe
- Analytical Validation
  - For each analyte the new reagents had excellent
    - linearity ($R^2 > 0.99$; slope 1.0 ± 0.05)
    - lower detection threshold (< 10 molecules)
    - Imprecision (CV < 10% > 100 molecules)
- External Standard Mixture (ESM)
  - Controls for inter-experimental variation including
    - Lot-to-lot variation in fluorometric probe intensity
    - Experimental variation in automatic cycle threshold selection

LCDT analysis of FFPE Samples

- **Opportunity:**
  - Large archives of formalin-fixed paraffin-embedded (**FFPE**) samples from subjects with known outcome and response to specific treatment.
  - Fine Needle Aspirate (**FNA**) is the most common method for diagnosing advanced lung cancer.



(dnavision.com)



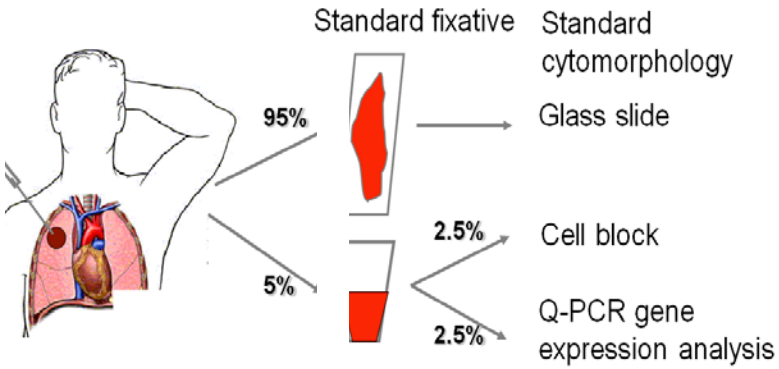
(www.mghradrounds.org)

- **Problem**: **Poor quality RNA from FFPE and FNA cell block FFPE samples.**
- **Challenge:**
  - Develop RT-PCR methods that reliably measure FFPE samples in which RNA is
    - Contaminated with interfering substances
    - Highly degraded
      - Analytical platform must be capable of analyzing small (<100 bp) PCR products

LCDT Analysis of Trans-Thoracic FNA Samples

Processing of Fine Needle Aspirate (FNA) Samples

- Most of needle aspirate ejected onto slide for cytomorphologic analysis
  - Cytomorphologic diagnosis is 75-85% accurate.
- Cells remaining in needle washed into FFPE solution for cell block
- **Opportunity:** Augment cytomorphologic diagnosis accuracy with molecular analysis.
- **Challenge:** Reliably measure DNA or RNA targets in small, degraded samples.
- **Solution:** Multiplex-competitive RT-qPCR in two rounds of amplification

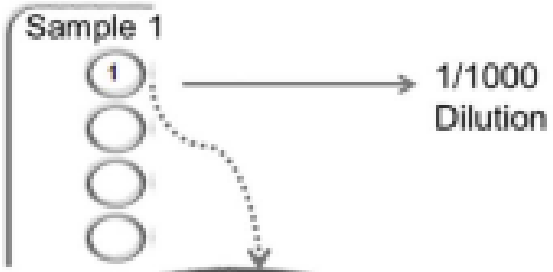


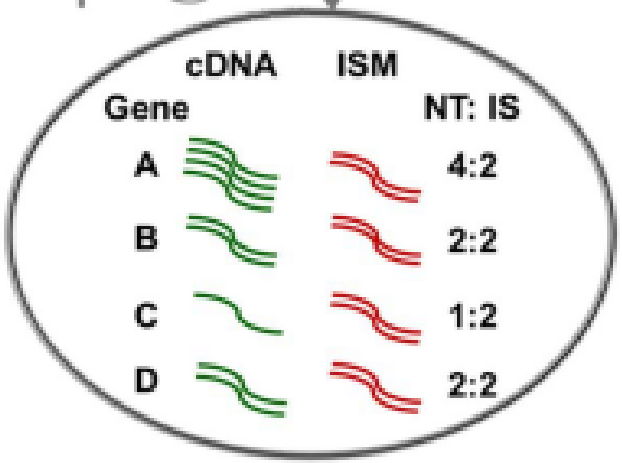
TWO COLOR FLUOROMETRIC ASSAYS

-Two rounds of PCR

1st round PCR

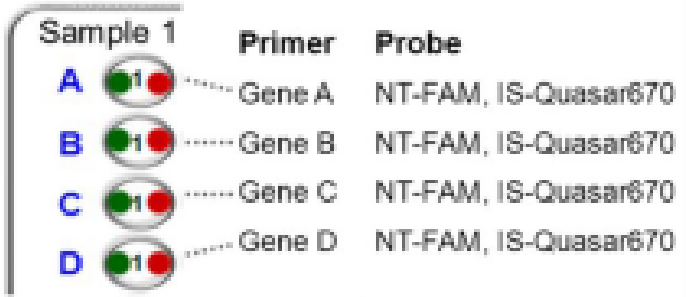
- Sample cDNA
- $6x10^3$ molec.IS (Gene A, B, C, D, n,..)
- Primer mix (Gene A, B, C, D, n,..)
- No probes
- 18 cycles

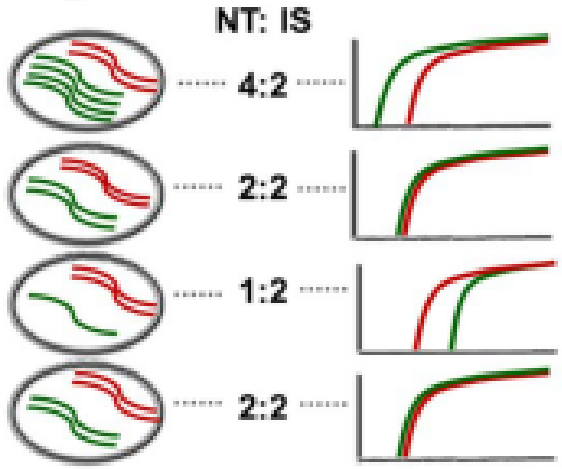




2nd round PCR

- Diluted 1st round product
- Primers for one gene
- Probes (NT and IS) for one gene
- 40 cycles

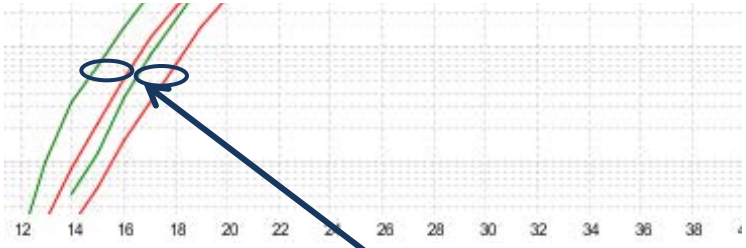




Experiment Set-Up in 96- Well Plate

- 20 samples: each mixed with aliquot of ISM, pre-amplified, and diluted 1,000-fold
- Measure each gene relative to known number of IS molecules within ISM
  - Measure four genes/sample
  - Measure each gene in ESM at 10^{-13} M and 10^{-14}M
  - No template control (control for false positives)

Two external standards plot (ACTB)

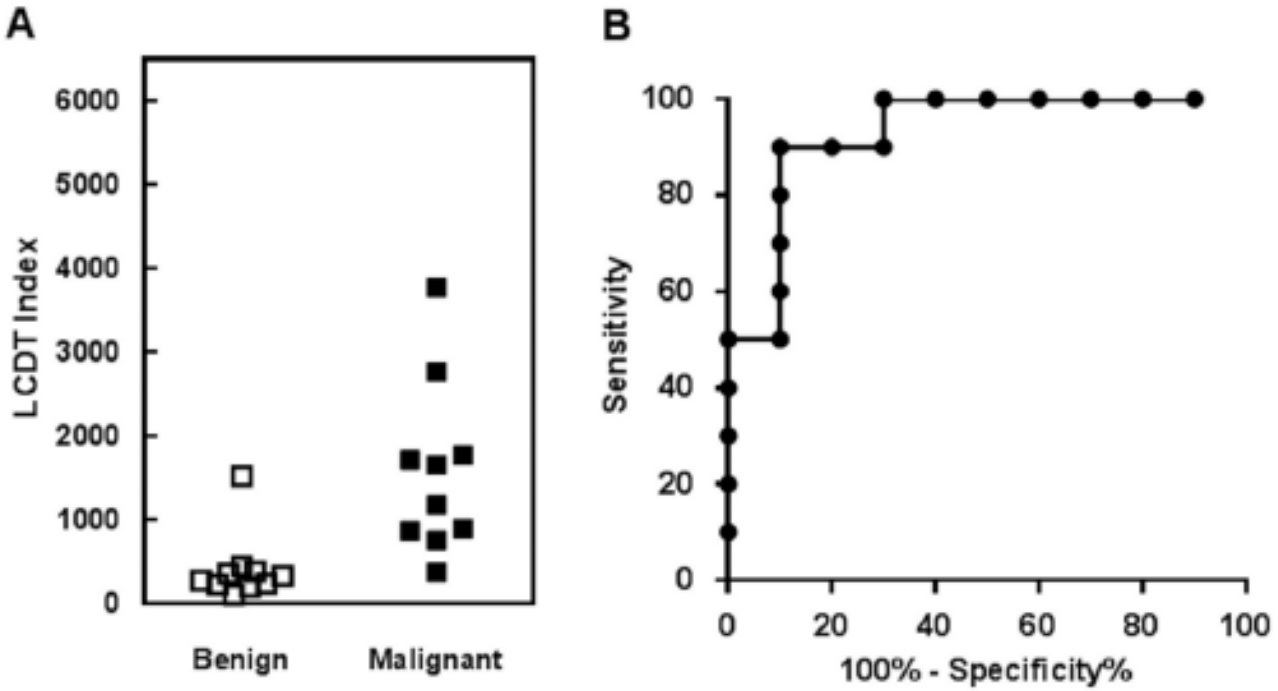


- **Sample 1**
  - **cDNA**
  - **ISM**
    - **600,000 molecules ACTB IS**
    - **60,000 molecules MYC IS**
    - **6,000 molecules E2F1 IS**
    - **6,000 molecules p21 IS**
  - **Sequence specific Probes**
    - **FAM probe for NT**
    - **QUASAR probe for IS**
  - **Buffer**
  - **dNTPs**
  - **Taq enzyme**

Assess 4 genes in 20 samples

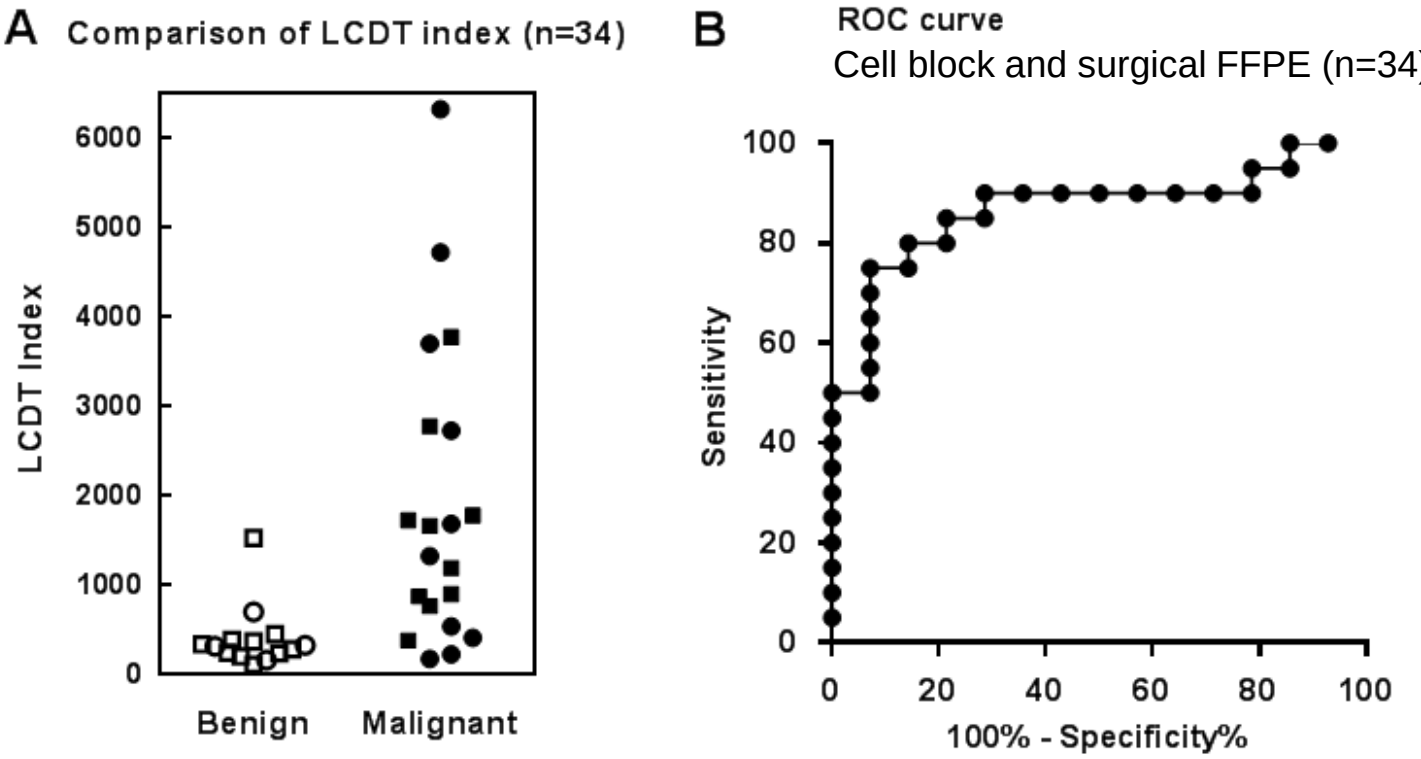
	1	2	3	4	5	6	7	8	9	10	11	12
ACTB	A	SM1	SM2	SM3	SM4	SM5	SM6	SM7	SM8	SM9	SM10	ESM 13M	ESM 14M
Myc	B	1	2	3	4	5	6	7	8	9	10	ESM	ESM
E2F1	C	1	2	3	4	5	6	7	8	9	10	ESM	ESM
CDKN1A	D	1	2	3	4	5	6	7	8	9	10	ESM	ESM
ACTB	E	SM11	SM12	SM13	SM14	SM15	SM16	SM17	SM18	SM19	SM20	NTC	NTC
Myc	F	11	12	13	14	15	16	17	18	19	20	NTC	NTC
E2F1	G	11	12	13	14	15	16	17	18	19	20	NTC	NTC
CDKN1A	H	11	12	13	14	15	16	17	18	19	20	NTC	NTC

LCDT Performance



- LCDT for surgical FFPE optimal cut-off value had **90% specificity** and **90% sensitivity**.
- Receiver Operator Characteristic (ROC) area under the curve (AUC) was **0.93** (confidence interval of 0.82-1.04)
- P-value 0.0061 for stratification malignant from non-malignant

LCDT Performance

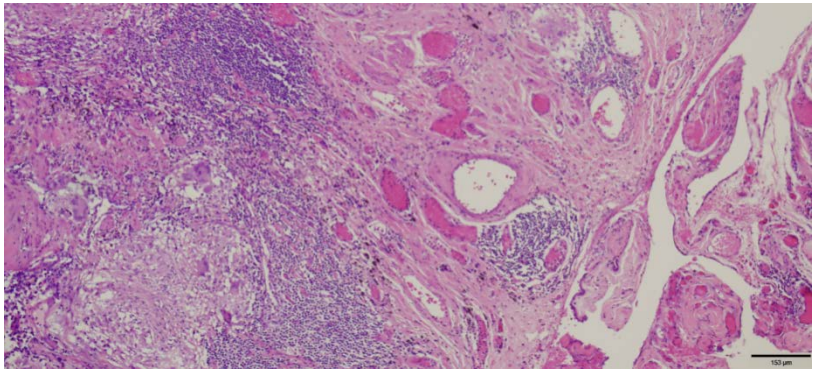


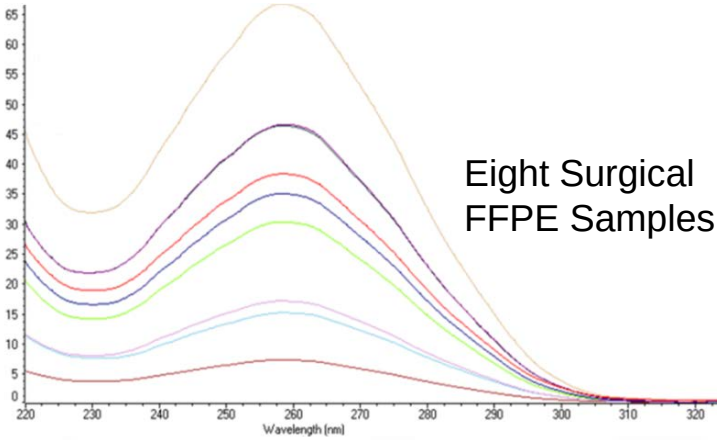
(A, Square: Surgical FFPE, Round: FNA FFPE)

- p-value of t-test for correct classification (Benign or Malignant) was **0.0009**.
- LCDT optimal cut-off value had **92.9% specificity** and **75% sensitivity**.
- Receiver Operator Characteristic (ROC) area under the curve (AUC) was **0.87** (confidence interval of 0.74 to 0.99).

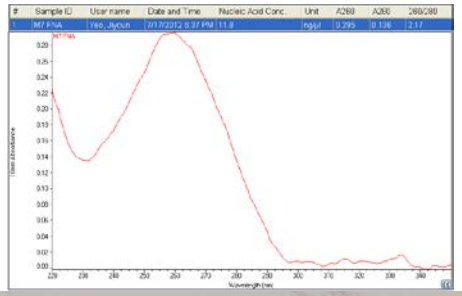
FFPE Sample characteristics

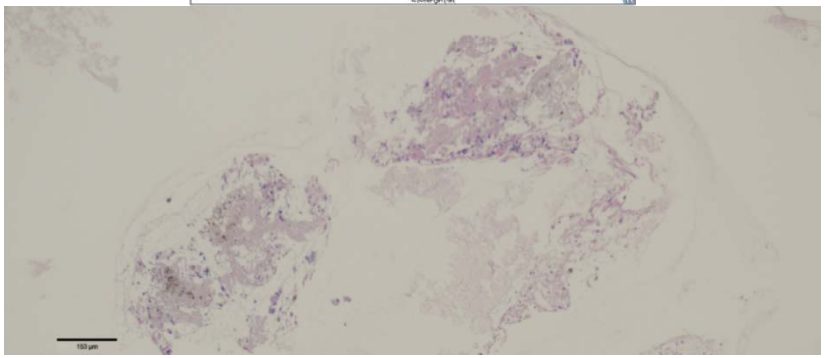
A) Surgical FFPE samples



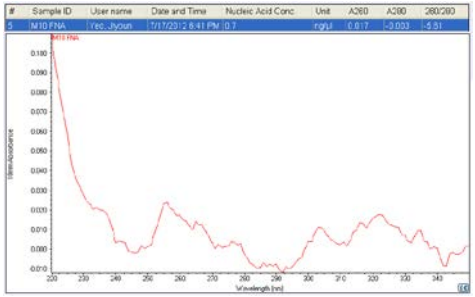


B) FNA FFPE samples





No bump at A260 & No smooth line



Poor FNA sample plot correlates with

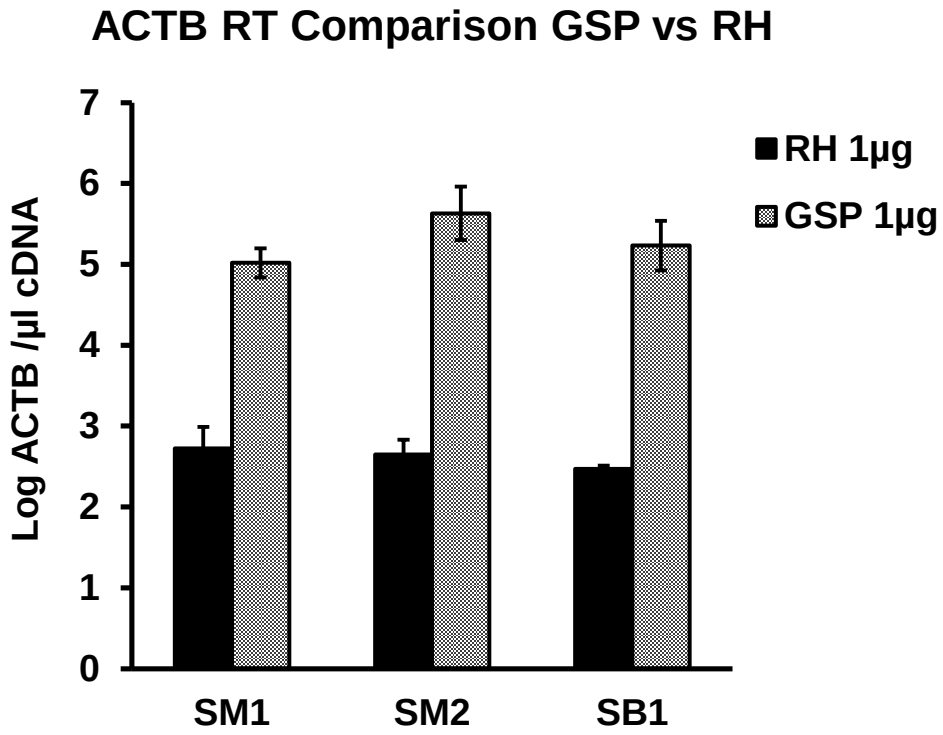
- RNA yield <150 ng
- Low cDNA yield following RT
- Failure of E2F1 measurement
- Higher CV & outlier measurements

FFPE RNA Extraction & Reverse Transcription

Low FFPE RNA Yield: Need to Maximize Signal

- Establish :
  - Optimal RNA extraction method
  - Optimal Reverse transcription conditions
  - Multiplex 2-round PCR

Comparison of Reverse Transcription (RT) by priming method



- 660-fold increase in yield of ACTB cDNA with gene specific primer (**GSP)** compared to random hexamer (**RH).**
- RT contained 1µg RNA from surgical FFPE sample SM1, SM2, or SB1.

LCDT Clinical Validation Plan

- LCDT test must be validated clinically in a prospective trial
- Plan to assess hundreds of transthoracic FNA cell block FFPE samples
  - Cytomorphologic analysis
  - LCDT analysis
- Compare sensitivity, specificity
- Assess ability of LCDT to augment accuracy of cytomorphology

Quantification of Fish Viral Hemorrhagic Septicemia virus (VHSv) with Two-Color Fluorometric Multiplex Competitive Real-Time PCR Assay

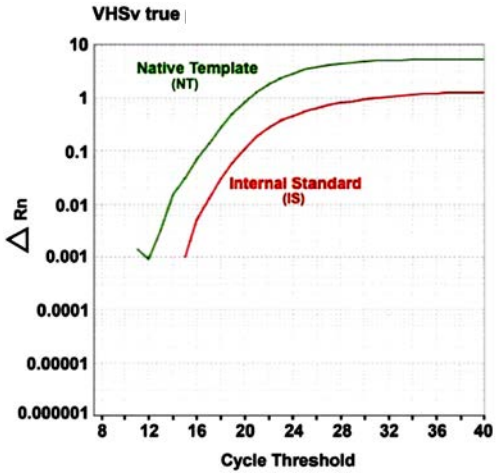
Pierce et all, PLoS ONE 8, 2013

Viral Hemorrhagic Septicemia virus (VHSv) is one of the world's most serious fish pathogens.

- There is need for faster, more accurate diagnostic test.

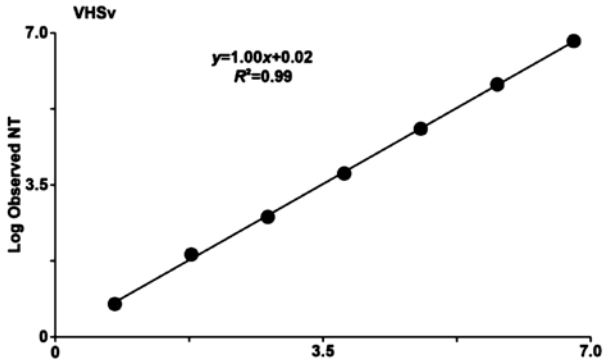
We developed a test using

- fish *actb1* gene as a loading control
- Internal standards for VHSv N-gene and *actb1* gene
- **FAM** probe for VHSv and actb1 NT
- **Quasar670** probe for each IS



Results demonstrate

- high signal-to analyte response (slope = 1.006)
- linear dynamic range spans seven orders of magnitude (R^2 = 0.99)



Conclusion

- This new VHSv assay is rapid, inexpensive, and has significantly greater accuracy than other published qRT-PCR tests and traditional cell culture diagnostics

VHSv Test application

- In preliminary studies, VHSv test threshold associated with fish symptoms/disease was identified.
- This threshold value will be evaluated in a larger study

AccuGenomics

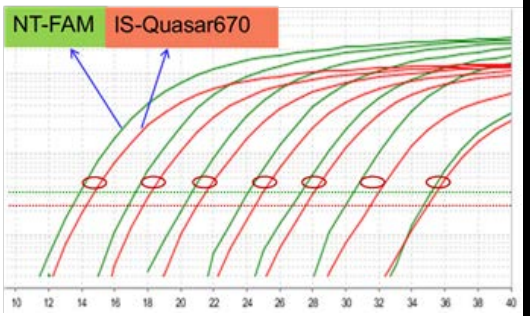
BIOSEARCH TECHNOLOGIES

Summary

For more robust measurement in highly degraded FFPE and FNA samples:

- Use competitive template Mixtures of Internal Standards (MIS) from Accugenomics, Inc. in combination with sequence-specific fluor-labeled hydrolysis probes from Biosearch Technologies, Inc.

  - Control for interfering substances
    - Prevent false negative results
  - **Enable** implementation on the commonly available real-time PCR thermocycler devices.
  - **Enable use of short PCR products (60-80bp)**
    - Reduce chance of a break in the RNA between the sequence regions primed for PCR.



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