FISH VALIDATION: HOW I DO IT!

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WHERE DO I GET THIS INFORMATION

- **American College of Medical Genetics**

  STANDARDS AND GUIDELINES FOR CLINICAL GENETICS LABORATORIES

  2009 Edition, Revised 01/2010

- ***New York State Department of Health Clinical Laboratory Standards of Practice***

- **Cytogenetics Checklist**

  CAP Accreditation Program

E9.2.1	**Validation requirements** vary with the regulatory status of the test/device (see above). Note that there may be alternative approaches to validation and the following is presented as a consensus method.

My validation cheat sheet

FISH Validation
Worksheet

Probe to Validate: _____

Manufacturer and order number: _____

Pre-Validation

- Brief description of the disease and the test
- Brief description of the affected gene(s) or region(s) and the nature and clinical relevance of the aberration(s)
- Indications for testing/reason for referral
- Bibliography of pertinent references. Include hard copies of references for uncommon tests
  - if article is an e-file save
- Specimen type(s) to be tested (cultured/uncultured, fixed/fresh); specify tumor types.
- Cell stage(s) to be tested
- Probe target, chromosomal band location, vendor, catalog number, and package insert
  tech: _____ date:_____
- Perform initial hybridization tests to determine optimal conditions
  - All testing parameters should be documented: tech: _____ date:_____
- Review results with supervisor sup: _____ date:_____
- Director review
  - Determine tissue type specificity: _____
  - Decide significance of signal patterns: _____
    _____ Dir: _____ date:_____

Validation

- Select 20-30 samples. Collect all relevant testing history of the samples selected.
- Insert reference numbers and tissue types on attached excel sheet
- Determine cells types targeted and scoring criteria
- Localization
  - Minimum of 5 metaphase cells from normal male- rev DAPI image with chromosomes labeled
- Sensitivity/ Specificity
  - 5 normal male samples- 100 metaphases (200 targets) analyzed min (95% and 98%)
- Accuracy
  - Compare FISH to G-banded analysis or another lab's results- 3 normal and 3 abnormal samples
- Normal Range
  - Min 20 or 30 normal specimens- 500 or 200 cells per specimen
  - Calculate cutoff values separately for each specimen/tissue type and signal pattern- betainv and SD
- Reproducibility
  - Intra- 3 samples tested in triplicate on 1 day
  - Inter- 2 additional replicates of sample tested on a different day

Scoring

- 2 readers/analyze 100 interphase cells looking at various hybridization patterns for the probe.
- A sample tracking list will be attached to this form listing all samples that require scoring.
- When a sample is complete please initial and date in the box next to the sample to confirm that sample is complete.

Data collection and statistics

- Data Entry tech: _____ date:_____
- Calculation of statistics: tech: _____ date:_____
- Supervisor review of data: sup: _____ date:_____
- Write validation summary tech: _____ date:_____

Final supervisor review: _____ Date: _____

Final director review: _____ Date: _____

☐ Creation of fragments and update reports if needed

WHY IS THE LAB VALIDATING THIS PROBE?

- Brief description of the disease and the test
- Brief description of the affected gene(s) or region(s) and the nature and clinical relevance of the aberration(s)
- Indications for testing/reason for referral
- Bibliography of pertinent references. Include hard copies of references for uncommon tests

E9.2	Documentation of **test or analytic validation** is required under CLIA '88 for any new test being placed into clinical service after September 1994. In the present context, a "test" is defined by the specific use of a probe, rather than by the generic "FISH" technology. Subsequent biannual **calibration** verification of the test system is also required under CLIA '88. (See 42 CFR §493.1217 and Section E9.3.)

WHAT SPECIMENS ARE BEING TESTED?

- Specimen type(s) to be tested (cultured/uncultured, fixed/fresh); specify tumor types- smears, direct, cultured, FFPE (not focus of this discussion)
- Cell stage(s) to be tested- interphase, metaphase, both
- Probe target, chromosomal band location, vendor, catalog number, and package insert- does your probe include gene of interest? MYC????
- Is there an internal control or does one need to be added
  - Two-color probes act as control (break-apart, fusion)
  - If single color probe- then add another color probe (8 centromere- I add 20q)
  - Don't forget about X/Y needing controls…
- Perform initial hybridization tests to determine optimal conditions
  - All testing parameters should be documented
  - Set up your spreadsheet
  - Make a FISH count sheet

At this point in time, I like to sit down with my lead/supervisor and review this data

Validation for CKS1B - 1q21 - Red / CDKN2C - 1p32 - Green, Amplification / Deletion Probe Set

				Positive signal patterns - Deletion				Negative signal patterns		Other signal patterns																
	Sample number	Specimen type	Preparation type	2R/1G	1R/1G	Total	Case Totals	2R/2G	Total	1R/2G	2R/3G	3R/2G ?	4R/2G	4R/4G	4R/3G	3R/3G	2R/5G	Total	Total cells scored	Difference between readers	Cytogenetic result	Flow cytometry result	Other assay result	Comments	Techs		
1	ISH11-004080	Mass	Touch preps	2		2	2	97	97	1								1	100	2	46,XY,add(1)(p36.3),t(14;18)(q32;q21)[2]/46,XY[18]	B-cell non-Hodgkin lymphoma of follicle center origin.	nuc ish(IGH,BCL2)x3(IGH con BCL2x2)[30/200]		AG		
				0		0		98	98	2								2	100						BC		
2	ISH11-004127	Lymph Node	Cultured cells	2		2	5	98	98									0	100	1	45,X,-Y,der(1)t(1;1)(p36.3;q21),t(3;9)(q27;p13)[5]/46,X,+X,-Y,t(3;9)[1]/46,XY[16]	There is no flow cytometric evidence for an abnormal or neoplastic lymphoid population in the analyzed sample.	nuc ish(BCL6,CDKN2A,CEP9)x2[200]	(False negative)	AR		
				2	1	3		97	97									0	100						AVB		
3	CG11-006083	Bone Marrow	Cultured cells	1		1	3	99	99									0	100	1	43,XX,-1,del(2)(p11.2p25),add(3)(q21),add(4)(q32),del(4) (p12p14), ?add(5)(q13),der(6)t(1;6)(p13;q21),-8,-8,-11, add(11)(p11.2),-12,-3,del(14)(q13q32),add(15)(p11.2), der(15)t(11;15)(q12;p11.2),add(16)(q22),-17, -18, i(18)(q10),+der(19)t(1;19)(q23;q13.3)x2,add(20)(q13.3), ?add(22)(p11.2),+r,+2-4mar[cp4]/46,XX[16]				ASM		
				2		2		97	97	1								1	100						JEB		
4	CG11-006809	Bone Marrow	Cultured cells			0	0	100	100									0	100	0	46,XY[20]	There is no flow cytometric evidence for an abnormal or neoplastic leukocyte population in the analyzed sample.			AVB		
						0		100	100									0	100						AG		
5	11V0057	Bone Marrow	Smear			0	0	100	100									0	100	0	46,XY[20]	There is no flow cytometric evidence for an abnormal or neoplastic leukocyte population in the analyzed sample.			HLS		
						0		100	100									0	100						FF		
6	11V0058	Bone Marrow	Smear	3		3	3	96	96									0	99	3	46,XY[21]	There is no evidence of acute myeloid leukemia, increased blasts, immunophenotypic features of dysmaturation on granulocytes/maturing myeloid cells, eosinophilia, or monocytosis. - There is no evidence of a lymphoproliferative disorder. - Hodgkin lymphoma cannot be confirmed or excluded by negative flow cytometry findings.			HLS		
						0		98	98			1				1		2	100						FF		
7	11V0061	Bone Marrow	Smear			0	0	99	99			1						1	100	0	46,XY[20]	Reversed myeloid to lymphoid ratio due to granulocytopenia and relative T-cell lymphocytosis. - There is no evidence of acute myeloid leukemia, increased blasts, immunophenotypic features of dysmaturation on granulocytes, eosinophilia, or monocytosis. - There is no evidence of a lymphoproliferative disorder.			BC		
						0		97	97					2		1		3	100						AR		
8	11V0062	Bone Marrow	Smear	2		2	2	98	98									0	100	2	46,XY[20]	Prominent eosinophilia. - There is no evidence of acute myeloid leukemia or increased blasts. - Granulocytes do not display overt immunophenotypic abnormalities. - There is no evidence of a B-cell lymphoproliferative disorder, atypical T-cell population, or monocytosis.			HLS		
						0		100	100									0	100						FF		

FISH Analysis Sheet – Mantle Cell Lymphoma

Patient Label

Tech Initials: _____ Date of Analysis: _____

Date of Processing: _____ Specimen: _____

							Quality		
Exported	Control approved	Probe / Analysis Area	Positive	Negative	Other	Total cells	Signal Strength	Background	Cellular Availability
		MYEOV,CCND1 11q13 Orange IGH 14q32 Green t(11;14)(q13;q32) **1%**							

☐ Entered data in LIS Initials of reporter's review: _____ Director's review: _____

Notes:

LOCALIZATION

	Localization should ensure that: the tested probe is the intended probe; no unknown probe is contaminating that lot Localization should also identify any cross-hybridization inherent to that probe. Probes with significant cross-hybridization should not be used.

Contamination: ***I personally have not seen this from commercial vendors; however, NEVER ASSUME***

Cross-hybridization: **This is a real issue. Hopefully worked out in optimization!**

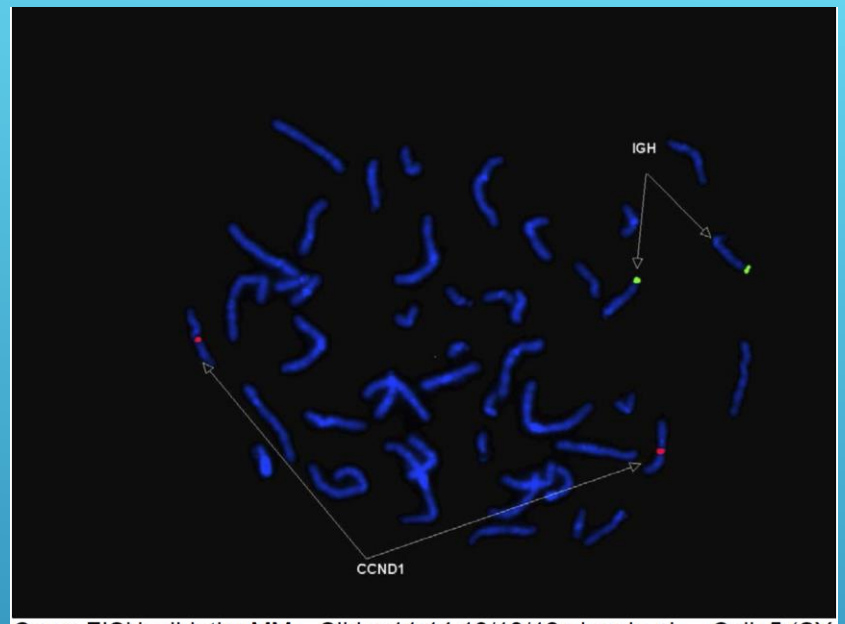
- **Centromeres are not unique- 15 centromere is notorious for x-hyb**
- **Have seen cross hybridization on metaphase FISH but NOT in interphase FISH**
  - **Document this in the validation write up and note that clinical tests will be performed on interphase nuclei**

LOCALIZATION cont.

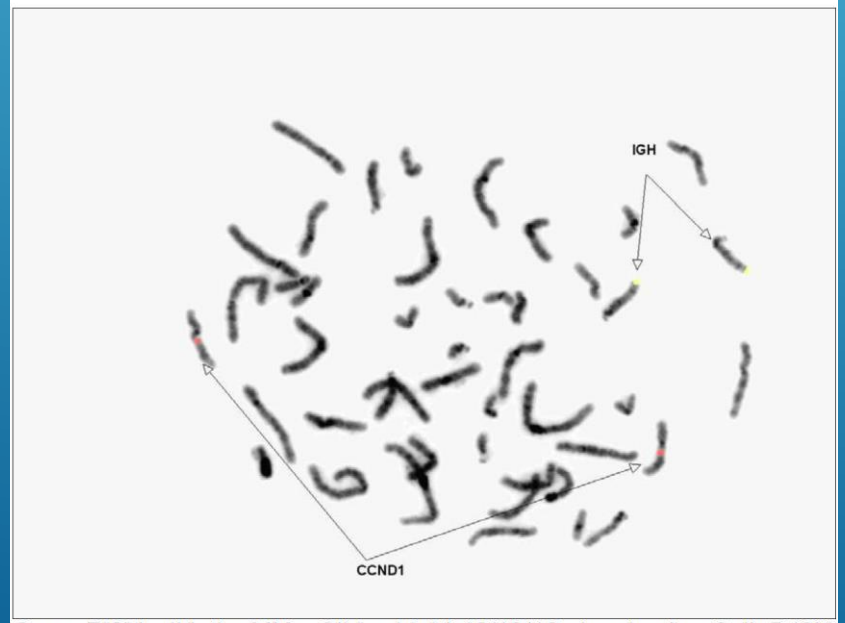
Minimum of 5 metaphase cells from normal male- rev DAPI image with chromosomes labeled

(Yes male – probe could cross-hyb to the Y chromosome too)
(Peripheral blood has the easiest chromosomes to localize with)

E9.2.1.1	Unique sequence FISH probes not approved as Class II or III ASR kits (ASRs, home-brews, IUOs and RUOs) must be validated in two ways: Probe **validation/localization** should be confirmed by: Scoring of a minimum of 5 metaphase cells to verify that each probe hybridizes to the appropriate chromosome target(s) and to no other chromosomes. Care should be taken in evaluation of potential probe contamination, as the contaminating probe may be present in a dilute concentration, thus hybridizing more weakly than the probe of interest. One of the following methods should be used to determine chromosomal localization: inverted DAPI, sequential G-/R-/or Q- to FISH or other banding method; use of a cell line containing the region of chromosome of interest as an independently identifiable target on a solid stained chromosome (e.g., structural rearrangements, trisomy, etc.); other methods that localize the probe at a level of resolution appropriate to the intended chromosome target. The source of the metaphase nuclei preparation may be any tissue type as the substrate (matrix) in question is the chromosome.

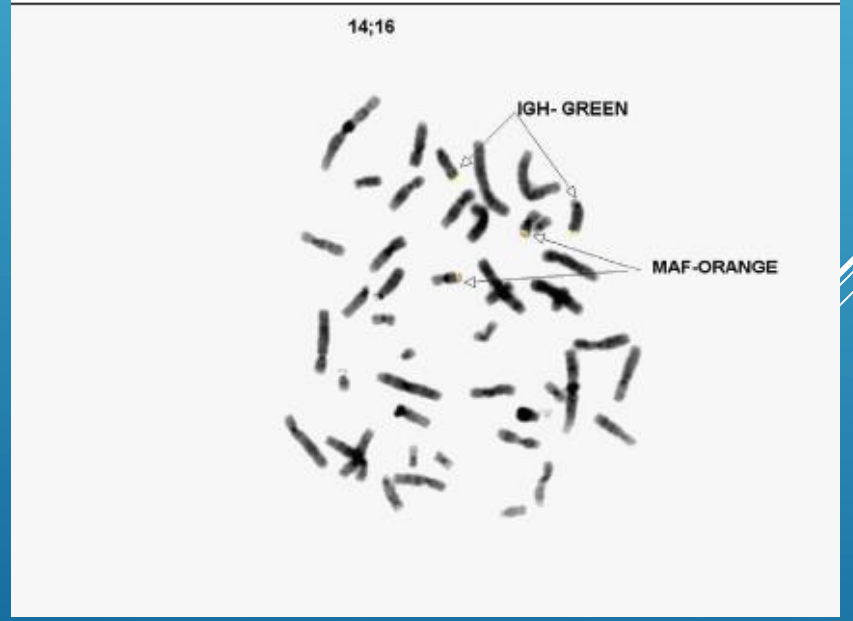


Case: FISHvalidationMM Slide: 11;14 10/10/12 nl male pb Cell: 5 (SY_



Case: FISHvalidationMM Slide: 11;14 10/10/12 nl male pb Cell: 5 (SY

Case: FISHvalidationMM Slide: igh/maf nl male pb Cell: 12K



SENSITIVITY AND SPECIFICITY

Analytical validation: sensitivity (percentage of scorable metaphase cells with appropriate number of distinct signals) and specificity (percentage of signals from nontarget sites) must be established for each new probe.

Assays using commercially available probes approved as ASR kits must meet the sensitivity and specificity parameters stated in package inserts provided by the manufacturer.

For probes that are not FDA approved for "in vitro diagnostic use," the laboratory must establish analytical sensitivity, specificity and reportable ranges of results.

Analytical sensitivity and specificity should be established by analysis of the hybridization of the probe to the chromosomes of interest (genomic target). Sensitivity is defined as the percentage of metaphases with the expected signal pattern at the correct chromosomal location. Specificity is defined by the percentage of signals that hybridize to the correct locus and no other location. An adequate number of cells and loci should be scored to ensure that the probe is sensitive and specific for the clinical testing being performed.

Sensitivity and specificity cont.

5 normal male samples-100 metaphases (200 targets) analyzed (minimum 95% and 98%)

I have always seen 100% for sensitive and specificity

Analytical sensitivity and specificity should be established by analysis of the hybridization of the probe to chromosomes representing at least 200 distinct genomic targets. For instance, a target sequence for which the hybridization signals from each of the chromatids is separable would require analysis of 50 cells (4 targets per metaphase). If the target sequence is at or near the centromere such that the hybridization signals are not clearly separable, the analysis would require 100 cells (2 targets per metaphase). Cells should be from 5 chromosomally characterized individuals (aneuploid cell lines can maximize target number). Pooling of cells from these 5 individuals (presuming comparable mitotic indices) is acceptable. However, all cells should have the same number of potential targets, if pooled. Discordance may require that the individual cell lines be tested separately. Greater discriminatory power may be needed to distinguish mosaicism.

Comparable analytic sensitivity and specificity must be established for each new lot of probe. This may be accomplished by analyzing a patient sample simultaneously with old and new lots to document consistency.

VALIDATION

- Select 30-40 control samples *(some always get thrown out)*
  - Collect all relevant testing history of the samples selected
- Get as many positives as you can- *positive patterns in cancer never follow the textbook*
- Insert reference numbers and tissue types on attached excel sheet

**In my experience leukemic blood and bone marrow give similar results, so I test them together*

E9.4	**Target tissues** include any tissue from which adequate numbers of dividing cells can be obtained. Although the above analytical validation considerations should be specific to the intended tissue of use, in tests that target metaphase chromosomes as the test matrix, the considerations are no different than those of standard cytogenetic analysis. Particular tissues and/or disease states may lead to chromosomes of poor morphologic quality and possibly to reduced signal intensity.
E10.3.1	**Database collection must be specific for an intended tissue type or cell population.** For instance, database collection must distinguish between cultured versus uncultured amniocytes due to known differences of cell populations. This differentiation between tissue types and cell populations is at the discretion of the laboratory director. The **normal database** should consist of an adequate number of cells from a group of control individuals (as determined by the director) who do not have abnormalities involving the target (and control) probes.Acceptable normal databases should include at least 500 nuclei each from 20 control samples or 200 nuclei each from 30 control samples.When possible, an **abnormal database** should be established. This database should be limited to include only samples from individuals who have abnormality(ies) involving the specific target probe(s).

POSITIVE SPECIMEN SPREADSHEET

	Validation for CKS1B - 1q21 - Red / CDKN2C - 1p32 - Green, Amplification / Deletion Probe Set,																								
				Positive signal patterns - Deletion					Negative signal patterns		Other signal patterns														
	Sample number	Specimen type	Preparation type	2R/1G	1R/1G ?	3R/1G	Total	Case Totals	2R/2G	Total	1R/2G	2R/3G	3R/2G	3R/3G	3R/0G	4R/0G	4R/1G		Total	Total cells scored	Difference between readers	Cytogenetic result	Flow cytometry result	Other assay result	Comments	Techs
1	val 001	Bone Marrow	Cultured cells			21	21	49	32	32			23		4	18	2		47	100	7	48-52,XY,-1,add(1)(q21),+del(3)(q21q27),+5,+add(6)(q27),+del(6) (q21q27)x2,+8,+add(9)(q34),-13,+19,-21,+2mar[cp5]/46,XY[16]				FF
						28	28		28	28			17		9	18			44	100						AR

VALIDATION cont.

- Determine cell types targeted and scoring criteria
  - (larger nuclei tend to be cancerous cells except when they are not/ "fried egg" plasma cells)
- Decide significance of signal patterns
- 2 Techs/analyze 100/250 interphase cells looking at various hybridization patterns for the probe.
  - *Remember to fill out training/competency forms for all techs analyzing probe type*
- Make note of unique findings regarding probe
  - *split signals, diffuse signals, sticky….*

E9.6.1	**Cell selection** for analysis should be based on the observed hybridization of the control probe(s) and the target-specific probe to metaphase chromosome(s). Cells showing chromosome-bound background (hybridization signals from nontarget sites) should not be scored.

E10.5.1	**Selection of cell nuclei** for analysis should be based on the observed hybridization of the probe(s). If utilizing a control probe, score only nuclei with the expected number of control probe signals. Nuclei that are broken, overlapped, or have significant background "noise" should not be scored.

Reproducibility

- Intra- 3 samples tested in triplicate on 1 day
- Inter- 2 additional replicates of sample tested on a different day

			Probes			
CELLS	REPLICATE	DATE	NFKB2	DATE	1P	1Q
LB022-MO	1	5/14/2013	99.0%	5/14/2013	98.5%	99.5%
	R1	5/28/2013	100.0%	5/28/2013	98.0%	97.5%
	R2	5/29/2013	98.5%	5/29/2013	100.0%	99.0%
	R3	5/29/2013	98.5%	5/29/2013	99.5%	99.5%
	R4	5/29/2013	98.0%	5/29/2013	99.5%	99.5%
	R5	5/30/2013	98.0%	5/30/2013	99.5%	100.0%
BMVAL-002	1	5/28/2013	98.0%	5/28/2013	100.0%	99.5%
	R1	5/28/2013	99.0%	5/28/2013	97.5%	97.0%
	R2	5/29/2013	99.0%	5/29/2013	100.0%	99.0%
	R3	5/29/2013	99.0%	5/29/2013	99.5%	99.5%
	R4	5/29/2013	96.5%	5/29/2013	99.5%	99.0%
	R5	5/30/2013	100.0%	5/30/2013	99.0%	97.5%
BMVAL005	1	5/28/2013	95.0%	5/28/2013	98.0%	97.5%
	R1	5/28/2013	95.5%	5/28/2013	99.5%	98.0%
	R2	5/29/2013	96.5%	5/29/2013	99.5%	97.5%
	R3	5/29/2013	97.5%	5/29/2013	97.5%	97.5%
	R4	5/29/2013	97.0%	5/29/2013	98.5%	99.5%
	R5	5/30/2013	97.5%	5/30/2013	98.5%	99.0%

DATA COLLECTION AND STATISTICS

- Double check data entry on spreadsheet
  - If >5% difference between techs- throw out or get third reader
- Using excel functions- determine cutoffs for the probe patterns
- Write validation summary
- Director signoff

	Averages:	1.583333	1	0.5				1.666667	2	1.166667	2	2	1	1	1	1
	Standard Deviation:	0.792961	#DIV/0!	0.905822				0.816497	0.707107	0.408248	#DIV/0!	0	#DIV/0!	0	#DIV/0!	1.300887
	3x Standard Deviation	2.378884	#DIV/0!	2.717465				2.44949	2.12132	1.224745	#DIV/0!	0	#DIV/0!	0	#DIV/0!	3.902662
	Average + 3x STD	3.962218	#DIV/0!	3.217465				4.116156	4.12132	2.391412	#DIV/0!	2	#DIV/0!	1	#DIV/0!	4.902662
Beta-inverse	Largest number	4	1		5			3	5	2	2	2	1	1	1	
	Beta-inverse	0.044306	0.023383		5%			0.03775	0.050596	0.030838	0.030838	0.030838	0.023383	0.023383	0.023383	
		(Needs to be largest number when reader 1 and reader 2 added together. So max(r1+r2) + 1 for beta-inverse calculation.)														

REFERENCES

Cancer Genet Cytogenet. 2008 May;183(1):1-5. doi: 10.1016/j.cancergencyto.2008.01.015.
Contemplations on preclinical validation of fluorescence in situ hybridization probe assay for paraffin-embedded tissues in hematologic disorders.
Reddy KS[1], Tripodi J.

Genet Med. 2006 Jan;8(1):16-23.
Preclinical validation of fluorescence in situ hybridization assays for clinical practice.
Wiktor AE[1], Van Dyke DL, Stupca PJ, Ketterling RP, Thorland EC, Shearer BM, Fink SR, Stockero KJ, Majorowicz JR, Dewald GW.

J Mol Diagn. 2009 Jul;11(4):330-3. doi: 10.2353/jmoldx.2009.080101. Epub 2009 Jun 12.
Statistical treatment of fluorescence in situ hybridization validation data to generate normal reference ranges using Excel functions.
Ciolino AL[1], Tang ME, Bryant R.