Clinical Validation of a Dickkopf-1 (DKK1) Chromogenic In-situ Hybridization (CISH) Assay for Manual and Image Analysis Assisted Pathologist Interpretation

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Background

Dickkopf-1 (DKK1) is a secreted modulator of Wnt signaling that is frequently overexpressed in tumors and associated with a poor prognosis. In this study, we demonstrate an approach for clinically validating a RNAscope chromogenic in-situ hybridization (CISH) assay for determining the level of DKK1 RNA in Gastric (G) and Gastroesophageal (GEJ) tumor tissues according to CLIA guidelines. This two-step process validated first the performance of the wet chemistry assay along with the ability of a pathologist to manually score the CISH signal according to a dot-based H-score paradigm, and second, the ability of an image analysis (IA) solution (Flagship Biosciences) to unbiasedly and reproducibly quantify DKK1 staining in the same set of samples.

Materials & Methods

Wet Chemistry Assay Validation: 20 G and 20 GEJ tumor formalin-fixed, paraffin-embedded samples (whole tissue resections) were stained for DKK1 RNAscope CISH with DKK1 (biomarker), PPIB (positive control for RNA integrity), and DapB (negative control) probes; 20 of the samples (10 G, 10 GEJ) were assessed with an orthogonal DKK1 qPCR assay for the accuracy assessment; 12 of the samples (6 G, 6 GEJ) had serial sections stained on 3 separate days for the precision assessment. Since the DKK1 RNAscope assay is valid only in PPIB positive tissues, a board-certified MD anatomical pathologist annotated PPIB expression regions of interest (ROIs) on digital images of the PPIB-stained tissues as reference for manual and digital DKK1 scoring. A board-certified MD pathologist provided the tumor H-score for all samples (see Fig. 1B). The DKK1 RNAscope CISH assay for manual pathology scoring was assessed for analytical specificity, sensitivity, accuracy, and precision (acceptance criteria: see Fig. 2C).

Image Analysis (IA) Solution Validation: The same DKK1-stained slides from the wet chemistry assay validation were scanned at 40X magnification and analyzed for the DKK1 RNAscope IA solution validation. Flagship's IA solution separated tumor and stromal compartments and quantified DKK1 expression within the PPIB positive ROIs. Resulting IA markups of cell detection and DKK1 expression were reviewed by a board-certified MD pathologist for acceptance (see Fig. 3). DKK1 staining was evaluated by IA to calculate the tumor H-score (see Fig. 4). The specificity, sensitivity, accuracy, and precision of the IA solution were examined (acceptance criteria: see Fig. 5C).

Results & Conclusions

The DKK1 RNAscope wet chemistry assay validation results (see Figs. 1 and 2) show that the DKK1 RNAscope assay yields high analytical sensitivity, specificity, accuracy, and precision in the determination of the manual pathology DKK1 H-score: > 90% of the tissue cohort met the passing criteria for sensitivity, specificity, and precision and the manual H-scores significantly correlated with the DKK1 qPCR assay for accuracy.

The DKK1 RNAscope IA solution validation results (see Figs. 4 and 5) show that the IA solution yields high analytical sensitivity, specificity, accuracy, and precision in the determination of the digital DKK1 H-score: ≥ 90% of the tissue cohort met the passing criteria for sensitivity, specificity, and precision and the digital and manual pathology H-scores were significantly correlated for accuracy.

These data demonstrate a clinically validated DKK1 RNAscope CISH laboratory-derived test (LDT) for manual and IA-assisted pathologist interpretation. This LDT is currently being used in a phase 2 clinical study of DKN-01, a DKK1 neutralizing antibody, in combination with tislelizumab to prospectively identify previously treated patients with elevated DKK1 tumor expression (Leap Therapeutics; NCT04363801).

The DKK1 RNAscope CISH Assay is Specific and Sensitive

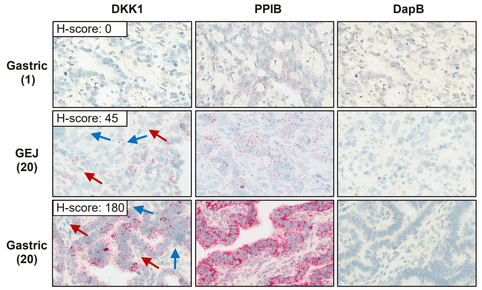


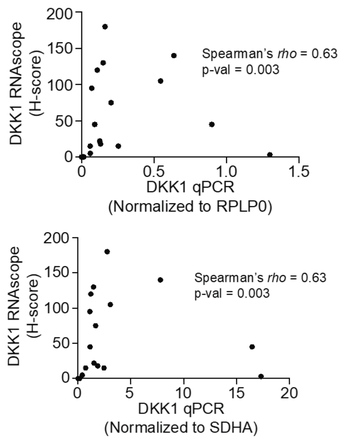
Fig. 1| A) DKK1 RNAscope Assay in 3 Representative Tumor Biopsies with No (top row), Moderate (middle row), and High (bottom row) DKK1 Signal Levels. DKK1 signal levels are shown with the PPIB positive control for RNA integrity and the DapB background negative control. Manual pathology H-scores are shown (see Fig. 1B). Red arrows: Non tumoral cells without DKK1 signal. Blue arrows: Tumor cells with low DKK1 signal (one dot/cell).

B) H-Score Scoring Paradigm for Quantification of DKK1 RNAscope Signal in Tumor Cells. DKK1 H-scores (range 0 to 300) were quantified using the following paradigm:

RNAscope Dot Quantification	Binned Score	Bin Label
No dots	0	Negative
1 to 3 dots/cell	1	Weak Positive
4 to 9 dots/cell	2	Medium Positive
10+ dots/cell	3	Strong Positive
Tumor H-Score = (%Bin0 * 0) + (%Bin1 * 1) + (%Bin2 * 2) + (%Bin3 * 3)		

The DKK1 RNAscope CISH Assay is Accurate and Precise

A. Validation of Accuracy of the DKK1 RNAScope Assay



B. Validation of Precision of the DKK1 RNAScope Assay

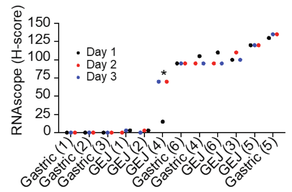


Fig. 2| Clinical Validation of the DKK1 RNAscope CISH Assay.
A) The DKK1 RNAscope assay is accurate as compared to the qPCR orthogonal method. Manual DKK1 RNAscope H-scores were significantly correlated to DKK1 qPCR results. Prior to RNA isolation for qPCR, regions with tumor cells were dissected from the biopsy for qPCR. qPCR data was normalized to housekeeping genes RPLP0 or SDHA.

B) The DKK1 RNAscope assay is precise. Replicate DKK1 staining was conducted on serial sections on three different days and manual H-scores were determined. All replicates were required to be in the same bin category or within a H-score of ± 20 if discordant binning occurred (see Fig. 2C). Asterisk indicates the one biopsy that failed precision.

C) The DKK1 RNAscope assay passed the acceptance criteria for analytical specificity, sensitivity, accuracy, and precision.

C. Summary of DKK1 RNAscope Validation Criteria and Results

Performance Parameter	Validation Acceptance Criteria	Cohort Pass/Fail	Cohort Percentage Pass
SPECIFICITY	**Sample Criteria:** Appropriate cell type and subcellular localization of DKK1 (i.e., tumor/malignant cells and occasional normal, inflammatory or stromal-associated cells), to include absence of staining in expected negative cells and/or subcellular compartment (as determined by the pathologist). **Cohort Criteria:** ≥ 90% of the tissue cohort must pass the sample criteria.	PASS	100% (40/40)
SENSITIVITY	**Sample Criteria:** Appropriate target tissue staining in expected cells and subcellular compartments, with a notable range of DKK1 signal expression across target cells in positive tissues. Background staining must be < 1 (Score 1 = 1-3 dots/cell) for acceptability (as determined by the pathologist). **Cohort Criteria:** ≥ 90% of the tissue cohort must pass the sample criteria.	PASS	100 % (40/40)
ACCURACY	**Sample Criteria:** Correlation between the manual pathology DKK1 tumor H-score and the qPCR (orthogonal method) average DKK1 threshold cycle (Ct) value (normalized to a housekeeping gene RPLP0 or SDHA) exhibits an acceptable concordance in 20 samples with a wide range of DKK1 expression. **Cohort Criteria:** Positive correlation with a statistically significant *p*-value (≤ 0.05; two-tailed).	PASS	*rho* = 0.629, *p* = 0.003 (RPLP0 or SDHA)
PRECISION	**Sample Criteria:** Concordance in binned DKK1 expression category according to the manual pathology DKK1 tumor H-score of the 3 sections from the sample stained on different days. In cases of discordant binning, a ± 20 point H-score discrepancy is still considered acceptable. DKK1 H-score Expression Bins Negative: H-score = 0 Low: H-score = 0+ to 34 High: H-score ≥ 35 **Cohort Criteria:** ≥ 80% of the tissue cohort must pass the sample criteria.	PASS	92% (11/12)

Cell Stratification and DKK1 Quantification Algorithms Approved by Pathologists

Clinical Data Acceptance Through Pathologist Integration with Image Analysis

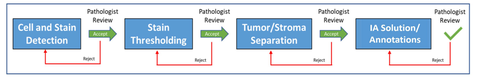


Fig. 3| Full Analytical IA Solution Development Workflow Integrates Pathologist Review for Clinical Oversight. As a quality control, checkpoints for pathology review are built into the IA solution development workflow process at multiple steps: accuracy of cell detection, stain thresholding for positivity and binned H-scores, tumor/stroma separation, and IA solution/annotation. Rejection by pathologist review results in algorithm refinement until pathologists can accept the data. Upon acceptance, the solution is locked and applied to the validation samples.

The DKK1 RNAscope Image Analysis Solution is Specific and Sensitive

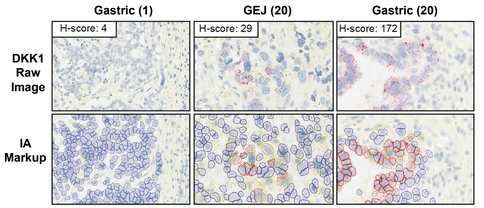


Fig. 4| DKK1 Image Analysis Markups in 3 Representative Tumor Biopsies with No (left column), Moderate (middle column), and High (right column) DKK1 Signal Levels. The raw tissue image from the DKK1 RNAscope CISH assay is shown (top row) alongside the same image with IA markups overlaid (bottom row). The DKK1 signal detected by the IA solution was specific for tumor cells. Cells with low DKK1 signal were detected by the IA solution indicating sensitivity. Blue IA markups: Cells with no DKK1 signal. Yellow IA markups: Cells with low DKK1 signal (1-3 dots). Orange IA markups: Cells with medium DKK1 signal (4-9 dots). Red IA markups: Cells with high DKK1 signal (10+ dots). The digital H-scores are shown and were calculated according to the same scoring paradigm used for manual pathology scoring (see Fig. 1B). Stromal cells were not highlighted by the algorithm and were not scored.

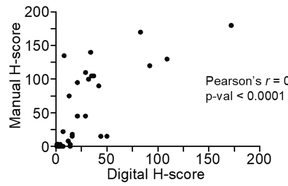
The DKK1 RNAscope Image Analysis Solution is Accurate and Precise

Fig. 5| Clinical Validation of the DKK1 RNAscope Image Analysis Solution. A) The DKK1 RNAscope IA solution is accurate as compared to the manual pathology orthogonal method. Manual and digital H-scores of the G/GEJ biopsies (*n* = 36) were significantly correlated. The 4 biopsies that did not pass the specificity assessment for the digital IA algorithm (see Fig. 5C) were excluded from the analysis.

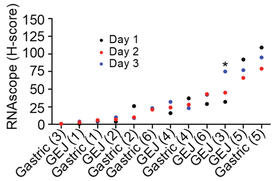
B) The DKK1 RNAscope IA solution is precise. Images from replicate DKK1 staining conducted on serial sections on different days were analyzed and digital H-scores were quantified. All replicates were required to be in the same bin category or within a H-score of ± 20 if discordant binning occurred (see Fig. 5C). Asterisk indicates a biopsy that did not meet the precision criteria.

C) The DKK1 RNAscope IA solution passed the acceptance criteria for analytical specificity, sensitivity, accuracy, and precision.

A. Validation of Accuracy of the DKK1 RNAScope IA solution



B. Validation of Precision of the DKK1 RNAScope IA solution



C. Summary of IA solution Validation Criteria and Results

Performance Parameter	Validation Acceptance Criteria	Cohort Pass/Fail	Cohort Percentage Pass
SPECIFICITY	**Sample Criteria:** Algorithm demonstrates appropriate cell recognition in ≥ 80% of cells evaluated, and the staining classification false positive rate is ≤ 20% (as determined by the pathologist). **Cohort Criteria:** ≥ 85% of the tissue image cohort must pass the sample criteria.	PASS	90% (36/40)
SENSITIVITY	**Sample Criteria:** Algorithm demonstrates appropriate cell identification in ≥ 80% of cells evaluated, and the staining classification false negative rate is ≤ 20% (as determined by the pathologist). **Cohort Criteria:** ≥ 85% of the tissue image cohort must pass the sample criteria.	PASS	100 % (40/40)
ACCURACY	**Sample Criteria:** Correlation between the digital DKK1 tumor H-score and the manual pathology DKK1 tumor H-score (orthogonal method) exhibits an acceptable concordance. **Cohort Criteria:** Positive correlation with a statistically significant *p*-value (≤ 0.05; two-tailed).	PASS	*rho* = 0.706, *p* < 0.0001; *r* = 0.740, *p* < 0.0001
PRECISION	**Sample Criteria:** Concordance in binned DKK1 expression category according to the digital DKK1 tumor H-score of the 3 sections from the sample stained on different days. In cases of discordant binning, a ± 20 point H-score discrepancy is still considered acceptable. H-score DKK1 Expression Bins Negative: H-score = 0 Low: H-score = 0+ to 34 High: H-score ≥ 35 **Cohort Criteria:** ≥ 80% of the tissue image cohort must pass the sample criteria.	PASS	92% (11/12)