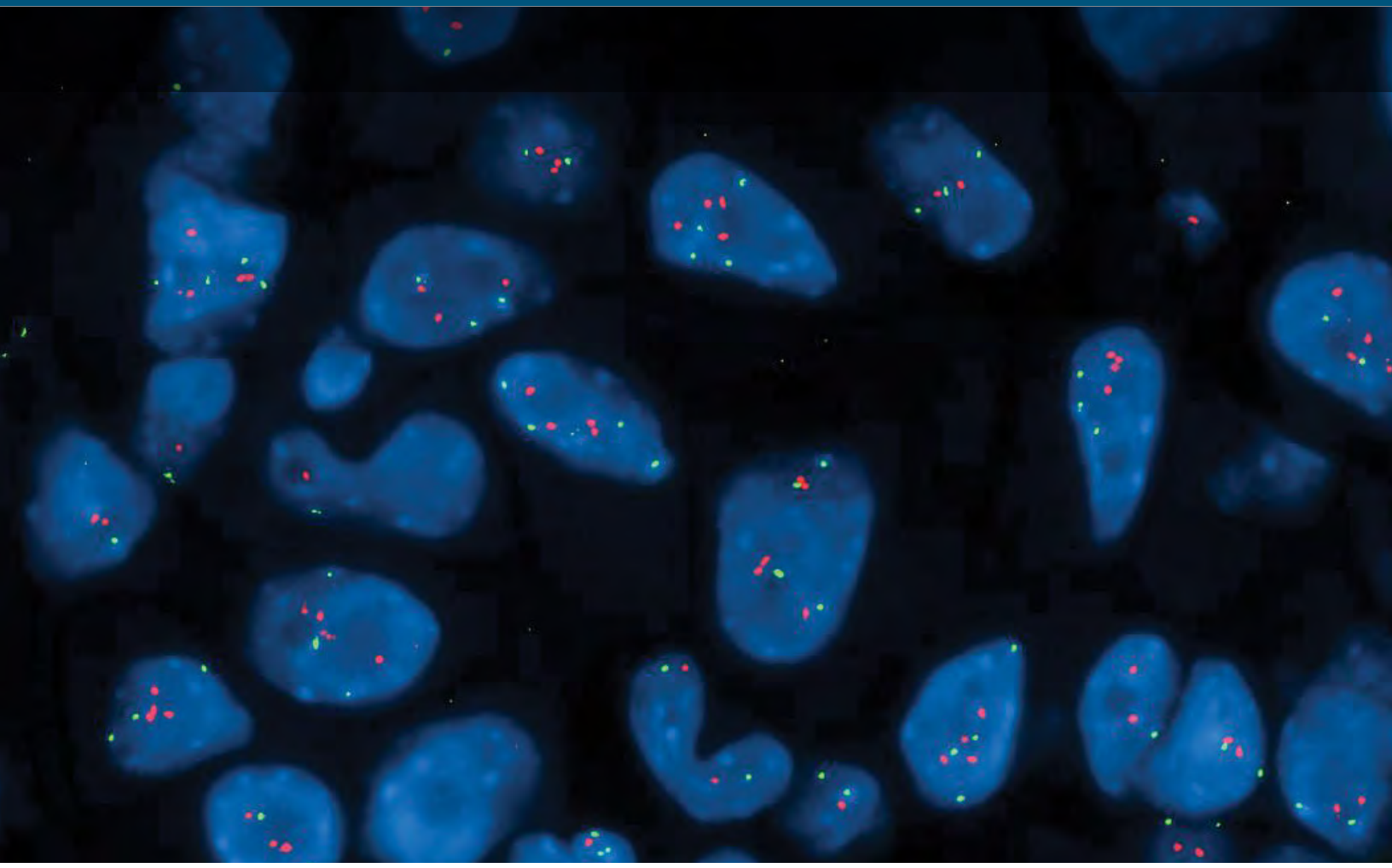
Agilent
Dako

Interpretation Guide
HER2 IQFISH pharmDx



Agilent
Trusted Answers

HER2 IQFISH pharmDx

HER2 IQFISH pharmDx is a direct fluorescence in situ hybridization (FISH) assay designed to quantitatively determine gene numeration in formalin-fixed, paraffin-embedded (FFPE) breast and gastric cancer tissue specimens.

Fast

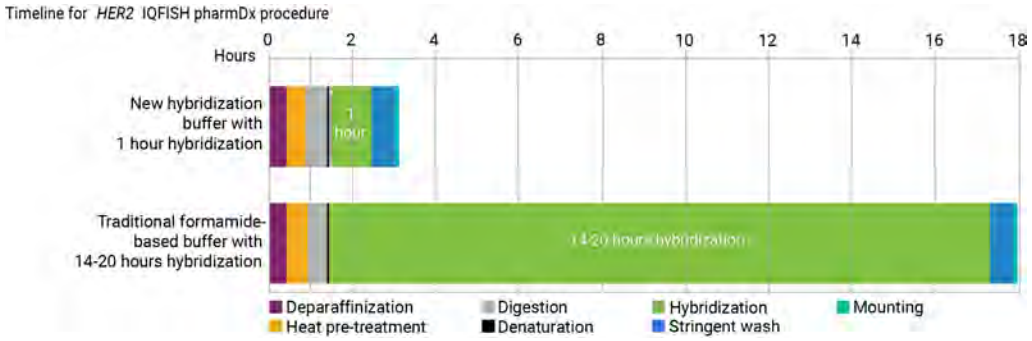
The *HER2* IQFISH pharmDx hybridization buffer reduces hybridization time to only 1-2 hours, enabling a turnaround time of only three hours and thirty minutes from dewax to counting.

Formamide Free

The *HER2* IQFISH pharmDx hybridization buffer is formamide free which eliminates the exposure of lab technicians to formamide.

Accurate

Robust protocol yields precise results with proven concordance to existing FDA-approved FISH assays.



Intended Use

HER2 IQFISH pharmDx is a direct fluorescence in situ hybridization (FISH) assay designed to quantitatively determine *HER2* gene amplification in formalin-fixed, paraffin-embedded (FFPE) breast cancer tissue specimens and FFPE specimens from patients with metastatic gastric or gastroesophageal junction adenocarcinoma.

HER2 IQFISH pharmDx is indicated as an aid in the assessment of breast and gastric cancer patients for whom Herceptin® (trastuzumab) treatment is being considered and for breast cancer patients for whom PERJETA™ (pertuzumab) or KADCYLA™ (ado-trastuzumab emtansine) treatment is being considered (see Herceptin®, PERJETA™ and KADCYLA™ package inserts).

For breast cancer patients, results from the *HER2* IQFISH pharmDx are intended for use as an adjunct to the clinicopathologic information currently used for estimating prognosis in stage II, node-positive breast cancer patients.

Filter Recommendation

Filters are individually designed for specific fluorochromes and for each microscope.

For the interpretation of *HER2* IQFISH pharmDx staining assays, the following combination of filters should be used:

- Specific DAPI filter
- High-quality Texas Red/FITC double filter (alternatively specific Texas Red and FITC single filters)

Fluorochrome	Excitation Wavelength	Emission Wavelength
FITC	495 nm	520 nm
Texas Red	596 nm	615 nm

Scoring Guide

Assessable Tissue

- In breast cancer score only the invasive component of a carcinoma
- In gastric cancer cases with intestinal metaplasia and adenocarcinoma in the same specimen, only the carcinoma component should be scored
- Avoid areas of heavy inflammation, necrosis and areas where the nuclear borders are ambiguous
- Disregard nuclei with weak signal intensity and non-specific or high background staining. Also disregard staining of bacterial DNA in mast cells and macrophages (highly red fluorescent cells that are clearly distinct from tumor cells with high gene amplification).

Quality Control

- Signals must be bright, distinct and easy to evaluate
- Normal cells allow for an internal control of the staining run
  - Normal cells should have 1-2 clearly visible green signals indicating that the CEN-17 PNA Probe has successfully hybridized to the centromeric region of chromosome 17
  - Normal cells should also have 1-2 clearly visible red signals indicating that the DNA Probe has successfully hybridized to the target region
  - Due to tissue sectioning, some normal cells will have less than the expected 2 signals of each color
  - Normal cells undergoing cell division may have more than the normal 1-2 signals of each color
  - Failure to detect signals in normal cells indicates assay failure, and results should be considered invalid
- Nuclear morphology must be intact when evaluated using a DAPI filter. Numerous ghost-like cells and a general poor nuclear morphology indicate overdigestion of the specimen, resulting in loss or fragmentation of signals. Such specimens should be considered invalid.
- Differences in tissue fixation, processing, and embedding in the user's laboratory may produce variability in results, necessitating regular evaluation of in-house controls

Breast Indication

Signal location

- Locate tumor on H&E-stained slide. Evaluate the same area on the *HER2* IQFISH-stained slide
- Scan several areas of tumor cells to account for possible heterogeneity
- Select distinct tumor areas for assessment
- Begin analysis in upper left quadrant of selected area. Scan from left to right, counting signals in each tumor cell nucleus.

Signal enumeration

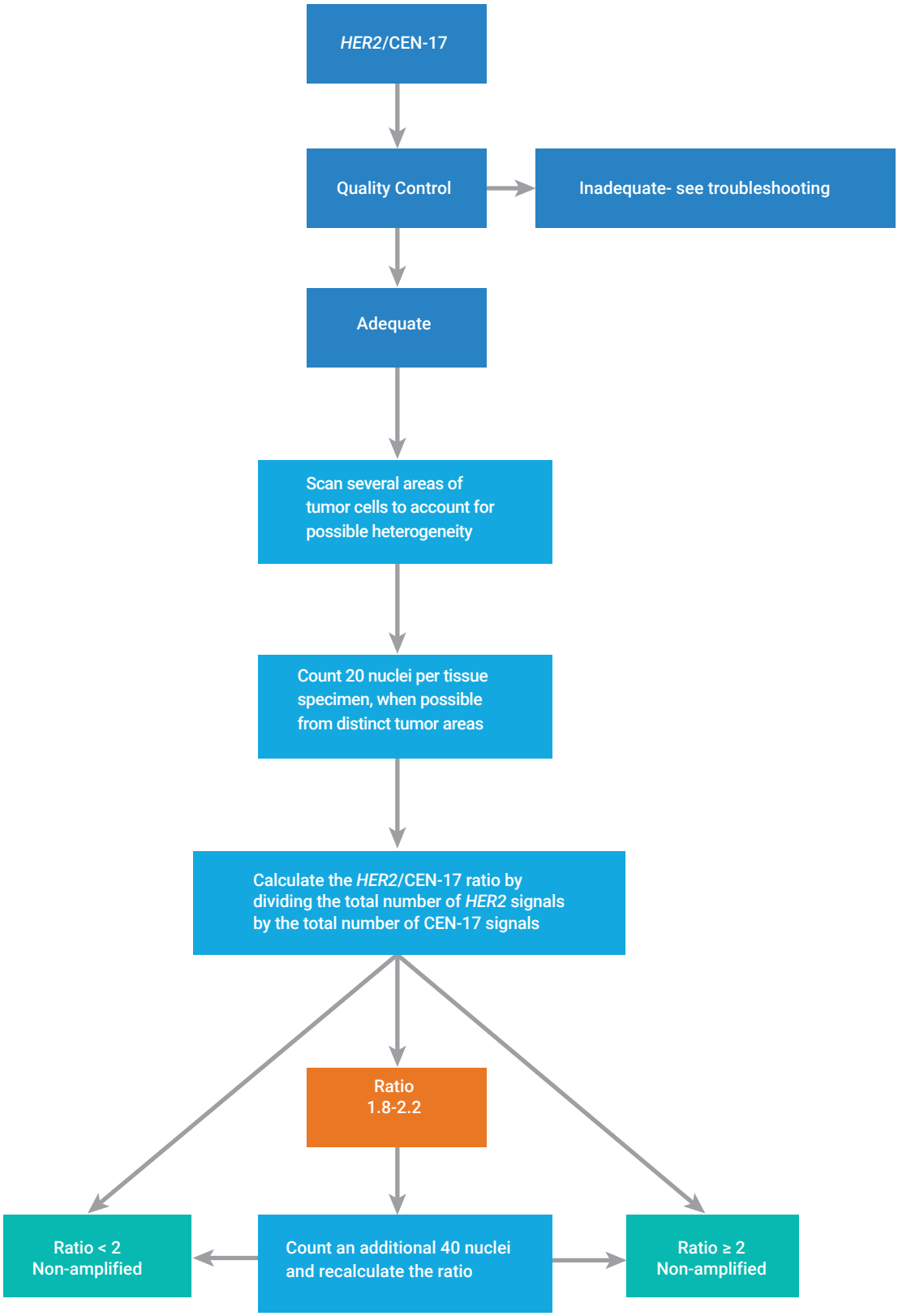
- Count 20 nuclei per tissue specimen, when possible from distinct tumor areas (see counting guide)
- Calculate the *HER2*/CEN-17 ratio by dividing the total number of *HER2* signals by the total number of CEN-17 signals
- Two signals of the same size, separated by a distance equal to or less than the diameter of one signal, are counted as one signal.
- Nuclei with high levels of *HER2* (red) gene amplification may exhibit formation of signal clusters. Estimate the *HER2* signal number. *HER2* clusters may obscure CEN-17 (green) signals. You can check this using a specific FITC filter.
- Nuclei exhibiting signals of only one color should not be scored
- Do not score nuclei demonstrating overdigestion
- Adjust microscope focus to locate all signals in the individual nuclei

Ratio of *HER2*/CEN-17 signals	*HER2* Gene Status	Result
< 2	Non-Amplified	Negative
≥ 2	Amplified	Positive

Results at or near the cut-off (1.8 – 2.2) should be interpreted with caution. In these cases, count an additional 20 nuclei and recalculate the ratio for the 40 nuclei.

For PERJETA™ 1 - In the randomized trial, *HER2* positivity was defined as being either IHC positive (3+) or FISH positive (HER2 CEN-17 ≥ 2.0) tested with HercepTest™, Dako or *HER2* FISH pharmDx Kit, Dako, respectively. Cases that were FISH negative but IHC positive (3+) were enrolled. Cases that were IHC negative (0, 1+) that were FISH positive (*HER2*/CEN-17 ≥ 2.0) were enrolled. However limited data (8/808) were available for cases that were positive only by FISH (no direct evidence of protein overexpression) and as such, caution should be exercised when interpreting these results.

For KADCYLA™ 2 - In the randomized trial, *HER2* positivity was defined as being IHC positive (3+) and/or FISH positive (*HER2*/CEN-17 ≥ 2.0). Cases that were FISH negative (*HER2*/CEN-17 <2.0) or unknown but IHC positive (3+) were enrolled (4.6%). Cases that were IHC negative (0, 1+) or unknown that were FISH positive (HER/CEN-17 ≥ 2.0) were enrolled (1.9%). The cases that were IHC positive and FISH positive made up 81.6% of the study population; the cases that were IHC equivocal (2+) and FISH positive cases made up 11.8%. However limited data (11/991) were available for cases that were positive by FISH but negative by IHC (no direct evidence of protein overexpression) and as such, caution should be exercised when interpreting the results.


HER2/CEN-17
Quality Control
Inadequate- see troubleshooting
Adequate
Scan several areas of tumor cells to account for possible heterogeneity
Count 20 nuclei per tissue specimen, when possible from distinct tumor areas
Calculate the *HER2*/CEN-17 ratio by dividing the total number of *HER2* signals by the total number of CEN-17 signals
Ratio 1.8-2.2
Ratio < 2 Non-amplified
Count an additional 40 nuclei and recalculate the ratio
Ratio ≥ 2 Non-amplified

Gastric Indication

Signal location

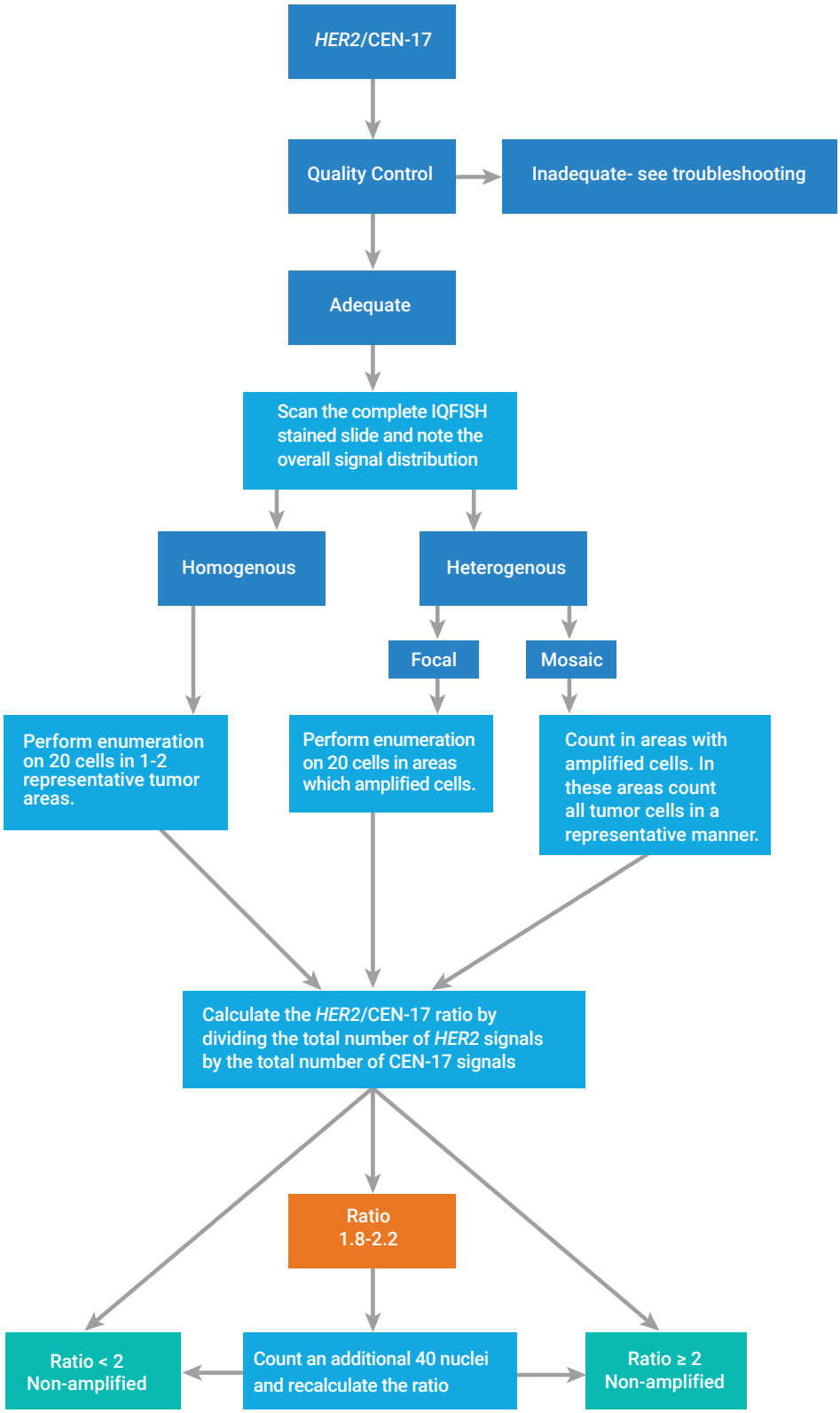
- Locate tumor on H&E-stained slide. Evaluate the same area on the IQFISH-stained slide
- Scan the complete IQFISH stained slide and note the overall signal distribution (homogeneous or heterogeneous)
- For homogeneous signal distributions, perform enumeration on 20 cells in 1-2 representative tumor areas
- For heterogeneous signal distribution, 20 cells are evaluated
  - If focal amplification exists, areas with amplified cells should be selected
  - If mosaic distribution exists, count in areas with amplified cells. In these areas, however, not only amplified cells should be counted, but rather all tumor cells in the area should be counted in a representative manner.
- When an area has been selected for signal evaluation, begin analysis in one of the 20 adjacent, chosen nuclei and then count in a cell-by-cell fashion excluding nuclei that do not meet the quality criteria.

Signal enumeration

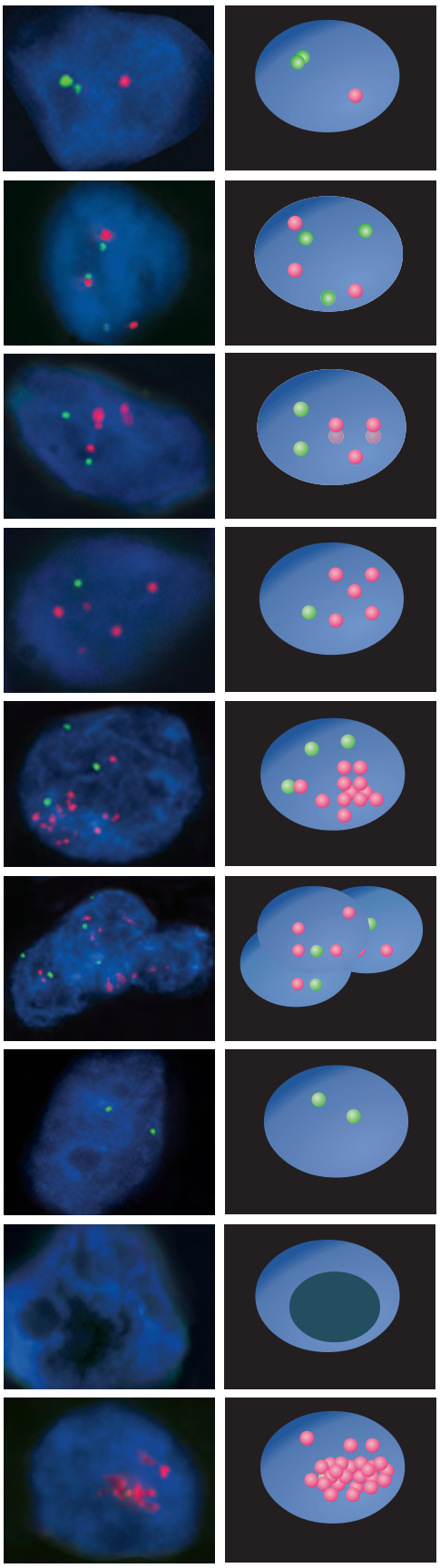
- Count *HER2* (red) and CEN-17 (green) signals in nuclei in representative tumor areas (see counting guide)
- Calculate the *HER2*/CEN-17 ratio by dividing the total number of *HER2* signals by the total number of CEN-17 signals
- Two signals of the same size, separated by a distance equal to or less than the diameter of one signal, are counted as one signal.
- Nuclei with high levels of *HER2* (red) gene amplification may exhibit formation of signal clusters. Estimate the *HER2* signal number. *HER2* clusters may obscure CEN-17 (green) signals. You can check this using a specific FITC filter.
- Nuclei exhibiting signals of only one color should not be scored
- Do not score nuclei demonstrating overdigestion
- Adjust microscope focus to locate all signals in the individual nuclei

Ratio of *HER2*/CEN-17 signals	*HER2* Gene Status	Result
< 2	Non-Amplified	Negative
≥ 2	Amplified	Positive

Results at or near the cut-off (1.8 – 2.2) should be interpreted with caution. In these cases, count an additional 40 nuclei and recalculate the ratio.


HER2/CEN-17
Quality Control
Inadequate- see troubleshooting
Adequate
Scan the complete IQFISH stained slide and note the overall signal distribution
Homogenous
Heterogenous
Focal
Mosaic
Perform enumeration on 20 cells in 1-2 representative tumor areas.
Perform enumeration on 20 cells in areas which amplified cells.
Count in areas with amplified cells. In these areas count all tumor cells in a representative manner.
Calculate the *HER2*/CEN-17 ratio by dividing the total number of *HER2* signals by the total number of CEN-17 signals
Ratio 1.8-2.2
Ratio < 2 Non-amplified
Count an additional 40 nuclei and recalculate the ratio
Ratio ≥ 2 Non-amplified

HER2 IQFISH pharmDx, Counting Guide



One green signal (split) indicates the presence of one copy of chromosome 17*. One red signal indicates the presence of one copy of the *HER2* gene.

The ratio of *HER2* to CEN-17 is 1/1 = 1; non-amplified.

Three green signals (one out of focus) indicate the presence of three copies of chromosome 17. Three red signals indicate the presence of three copies of the *HER2* gene.

The ratio of *HER2* to CEN-17 is 3/3 = 1; non-amplified.

Two green signals indicate the presence of two copies of chromosome 17. Three red signals (two split signals) indicate the presence of three copies of the *HER2* gene*.

The ratio of *HER2* to CEN-17 is 3/2 = 1.5; non-amplified.

One green signal indicates the presence of one copy of chromosome 17. Five red signals indicate the presence of five copies of the *HER2* gene.

The ratio of *HER2* to CEN-17 is 5/1 = 5; amplified

Three green signals indicate the presence of three copies of chromosome 17. Approximately 12 red signals indicate the presence of 12 copies of the *HER2* gene (cluster estimation).

The ratio of *HER2* to CEN-17 is 12/3 = 4; amplified.

Do not score (nuclei are overlapping, not all areas of nuclei are visible).

Do not score nuclei with signals of only one color (two green signals).

Do not score (overdigested nuclei).

Cluster of red signals hiding green signals. Check the green signals with a specific FITC filter, or do not score

**Two signals of the same size, separated by a distance equal to or less than the diameter of one signal, are counted as one signal*

Troubleshooting Guidelines for *HER2* IQFISH pharmDx

For further troubleshooting help, contact your local Agilent representative.

Problem	Probable Cause	Suggested Action
No signals or weak signals	Kit has been exposed to high temperatures during transport or storage	Check storage conditions. Ensure that dry ice was present when the consignment was received. Ensure that vial 3 has been stored at -18 °C in the dark. Ensure that vials 2A and 5 have been stored at maximum 2-8 °C, and that vials have been stored in the dark.
	Microscope not functioning properly - Inappropriate filter set - Improper lamp - Mercury lamp too old - Dirty and/or cracked collector lenses - Unsuitable immersion oil	Check the microscope and ensure that the used filters are suitable for use with the kit fluorochromes, and that the mercury lamp is correct and has not been used beyond expected lifetime. In case of doubt, please contact your local microscope vendor.
	Faded signals	Avoid long microscopic examination and minimize exposure to strong light sources.
	Pre-treatment conditions incorrect	Ensure that the recommended pre-treatment temperature and time are used.
	Evaporation of Probe Mix during hybridization	Ensure sufficient humidity in the hybridization chamber
No red signals	Pre-treatment conditions incorrect	Ensure that the recommended pre-treatment temperature and time are used
Areas without signal	Probe volume too small	Ensure that the probe volume is large enough to cover the area under the coverslip
	Air bubbles caught during Probe Mix application or mounting	Avoid air bubbles. If observed, gently tap them away using forceps
Excessive background staining	Inappropriate tissue fixation	Ensure that only formalin-fixed, paraffin-embedded tissue sections are used
	Paraffin incompletely removed	Follow the deparaffinization and rehydration procedures outlined in Section B.2
	Stringent wash temperature too low	Ensure that the stringent wash temperature is 63 (±2) °C
	Prolonged exposure of hybridized section to strong light	Avoid long microscopic examination and minimize exposure to strong light
Poor tissue morphology	Incorrect Pepsin treatment	Adhere to recommended Pepsin incubation times. See section B.3, step 2. Ensure that the Pepsin is handled at the correct temperature. See Section B.1.
	Incorrect pre-treatment conditions may result in unclear or cloudy appearance	Ensure that the recommended pre treatment temperature and time are used
	Too long Pepsin treatment or very thin section thickness may cause ghost cells or donut cells to appear.	Shorten the Pepsin incubation time. See section B.3, step 2.Ensure that the section thickness is 3-6 µm.
High level of green auto fluorescence on slide including areas without FFPE tissue	Use of old glass slides	Ensure that the coated glass slides (Dako Silanized Slides, Code S3003, or poly-L lysine-coated slides) have not passed expiry date.

Note

If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please call Agilent Technical Support for further assistance.

Scoring Sheet

HER2 IQFISH pharmDx Kit, Code K5731 - Breast Indication

Date of Run: _____

HER2 IQFISH pharmDx Kit K5731 Lot: _____

Staining Run Log ID: _____

Specimen ID: _____

Count signals in 20 tumor nuclei

Nucleus no.	*HER2* Score (Red)	CEN-17 Score (Green)	Nucleus no.	*HER2* Score (Red)	CEN-17 Score (Green)
1			11		
2			12		
3			13		
4			14		
5			15		
6			16		
7			17		
8			18		
9			19		
10			20		
Total (1-10)			Total (11-20)		

For determination of the *HER2*/CEN-17 ratio, count the number of *HER2* signals and the number of CEN-17 signals in the same 20 nuclei and divide the total number of *HER2* signals by the total number of CEN-17 signals. If the *HER2*/CEN-17 ratio is borderline (1.8 –2.2), count an additional 20 nuclei and recalculate the ratio.

A ratio at or near the cut-off (1.8-2.2) should be interpreted with caution (see counting guide).

	HER2	*CEN-17*	*HER2/CEN-17 ratio*
TOTAL SCORE(1-20)			

☐ Ratio **< 2:** *HER2* gene amplification was not observed

☐ Ratio **≥ 2:** *HER2* gene amplification was observed

Technician signture: _____ Date_____

Pathologist signature: _____Date_____

Scoring Sheet

HER2 IQFISH pharmDx Kit, Code K5731 - Gastric Indication

Date of Run: _____

HER2 IQFISH pharmDx Kit K5731 Lot: _____

Staining Run Log ID: _____

Specimen ID: _____

Characterization of signal distribution in tissue: _____

Homogeneous: ☐ Heterogeneous - Focal: ☐ or Heterogeneous - Mosaic: ☐

Count signals in 20 tumor nuclei

Nucleus no.	*HER2* Score (Red)	CEN-17 Score (Green)	Nucleus no.	*HER2* Score (Red)	CEN-17 Score (Green)
1			11		
2			12		
3			13		
4			14		
5			15		
6			16		
7			17		
8			18		
9			19		
10			20		
Total (1-10)			Total (11-20)		

For determination of the *HER2*/CEN-17 ratio, count the number of *HER2* signals and the number of CEN-17 signals in the same 20 nuclei and divide the total number of ***HER2*** signals by the total number of CEN-17 signals. If the *HER2*/CEN-17 ratio is borderline (1.8 –2.2), count an additional 40 nuclei and recalculate the ratio (refer to recount scoring scheme). A ratio at or near the cut-off (1.8-2.2) should be interpreted with caution (see counting guide).

HER2 FISH	*HER2*	*CEN-17*	*HER2/CEN-17 ratio*
TOTAL SCORE(1-20)			

☐ Ratio ***< 2:*** *HER2* gene amplification was not observed

☐ Ratio ***≥ 2:*** *HER2* gene amplification was observed

Technician signture: _____ Date_____

Pathologist signature: _____Date_____

HER2 IQFISH pharmDx Kit, Code K5731 - Gastric Indication

Recount Scoring Scheme

Date of Run: _____

HER2 IQFISH pharmDx Kit K5731 Lot: _____

Staining Run Log ID: _____

Specimen ID: _____

Count signals in 20 tumor nuclei

Nuclei no.	Red *HER2*	Green CEN-17	Nuclei no.	Red *HER2*	Green CEN-17	Nuclei no.	Red *HER2*	Green CEN-17	Nuclei no.	Red *HER2*	Green CEN-17
1			11			21			31		
2			12			22			32		
3			13			23			33		
4			14			24			34		
5			15			25			35		
6			16			26			36		
7			17			27			37		
8			18			28			38		
9			19			29			39		
10			20			30			40		
Total 1-10			Total 11-20			Total 21-30			Total 31-40		

For determination of the *HER2*/CEN-17 ratio, count the number of *HER2* signals and the number of CEN-17 signals in the same 40 nuclei and divide the total number of *HER2* signals by the total number of CEN-17 signals. Report Total Score from the 1-40 nuclei in the table below.

HER2 FISH	*HER2*	*CEN-17*	*HER2/CEN-17 ratio*
TOTAL SCORE (1-40)			

☐ Ratio ***< 2:*** *HER2* gene amplification was not observed

☐ Ratio ***≥ 2:*** *HER2* gene amplification was observed

Technician signture: _____ Date_____

Pathologist signature: _____Date_____

HER2 IQFISH pharmDx Kit

- *HER2*/CEN-17 IQFISH Probe Mix
- Pre-Treatment Solution (20x concentrated)
- Pepsin, Ready-to-Use
- Pepsin Diluent (10x concentrated)
- Stringent Wash Buffer (20x concentrated)
- Fluorescence Mounting Medium, containing DAPI
- Wash Buffer (20x concentrated)
- Coverslip Sealant

***HER2* - Related Products**	**Size**	**Code**
HER2 IQFISH pharmDx	20 tests	K5731
Histology FISH Accessory Kit	20 tests	K5799
HercepTest™ (for manual use)	35 tests	K5204
HercepTest™ for the Dako Autostainer	50 tests	K5207
HercepTest™ for Automated Link Platforms	50 tests	SK001

Notes

Notes

References

1. Baselga J, Cortés J, Kim SB, Im SA, Hegg R, Im YH, et al. Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. N Engl J Med 2012;366:109-19.
2. A Phase III, Randomized, Multicenter, Open-label Study of the Efficacy and Safety of Trastuzumab-MCC-DM1 vs. Capecitabine + Lapatinib in Patients with *HER2*-Positive Locally Advanced or Metastatic Breast Cancer Who Have Received Prior Trastuzumab-based Therapy

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