

Standardisation of EGFR FISH in colorectal cancer: results of an international interlaboratory reproducibility ring study

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ABSTRACT

Aims Epidermal growth factor receptor (*EGFR*) gene copy number evaluated by fluorescence in situ hybridisation (FISH) can discriminate among *KRAS* wild-type patients those with better outcome to *EGFR*-targeted therapy in metastatic colorectal cancer, further enhancing selection of patients. Nevertheless, enumeration of gene copies is challenging and the lack of analytical standardisation has limited incorporation of the test into the clinical practice. We therefore assessed *EGFR* FISH interlaboratory consensus among five molecular diagnostic reference centres.

Methods A set of 12 colorectal cancer samples circulated among laboratories, and samples were scored according to commonly agreed guidelines.

Reproducibility was quantified using the standard error of measurement (SEM).

Results A SEM of 0.865 and a within-subject coefficient of variation (WSCV) of 26.8% for mean *EGFR* gene/nuclei and a SEM of 0.235 and a WSCV of 19.4% for the mean *EGFR* gene/CEP7 ratio were observed. Measurement of the fraction of cells displaying chromosome 7 polysomy showed WSCV of 46.6%, 34.0% and 51.0% for percentage of cells displaying ≤ 2 , ≥ 3 and ≥ 4 *EGFR* signals, respectively. Among different slides of the same specimen, the WSCV was 6.1% for mean *EGFR* gene/nuclei and 3.9% for mean of *EGFR* gene/CEP7 ratios.

Conclusions Molecular diagnosis of *EGFR* gene copy number by FISH varied largely among pathology centres, with fluctuations covering the whole range of proposed cut-offs of predictive usefulness from literature.

Definition of a detailed scoring system and implementation of comprehensive training programmes for laboratories are therefore necessary before including the test into clinical practice.

INTRODUCTION

Targeted therapy of metastatic colorectal cancer (mCRC) with anti-epidermal growth factor receptor (*EGFR*) monoclonal antibodies (moAbs) cetuximab or panitumumab has been revolutionised by the introduction of genetic profiling of individual tumours. Although initial response rates of about 10% were seen in patients with chemorefractory mCRC, it was subsequently discovered that higher response rates in the range of 13%–17% were achievable in tumours without mutations in codon 12 or 13 of the *KRAS* gene, whereas only 0%–1.2% of the *KRAS* mutant tumours responded

to therapy.^{1 2} Nevertheless, even in *KRAS* wild-type CRCs, about 40% of the previously untreated^{3–5} and about 60%–70% of the previously treated^{6 7} do not respond to anti-*EGFR* treatment and additional detection of *NRAS*, *BRAF* and *PIK3CA* exon 20 mutations⁸ and loss of PTEN protein⁹ or better discrimination among *KRAS* mutations by excluding G13D carriers¹⁰ may further enhance selection of patients.

In addition to these negative predictive molecular alterations, the *EGFR* gene copy number (GCN) stood up as a candidate biomarker for predicting response of CRC to anti-*EGFR* therapy.^{7 11} *EGFR* GCN could indeed further discriminate among *KRAS* wild-type patients those better candidates to cetuximab or panitumumab, enhancing patients' selection by achieving response rates as high as 80%.^{12–14} This notion has been recently supported also by a study in which a molecularly annotated platform of patient-derived xenografts ('xenopatient') was exploited for identifying novel mechanisms of resistance to cetuximab, confirming that *EGFR* GCN gain (as assessed by quantitative PCR) tended to segregate responders also in this preclinical context.¹⁵ Fluorescence in situ hybridisation (FISH) has been used almost invariably in retrospective clinical studies for assessing *EGFR* GCN in CRC.^{11 13 14} However, signal enumeration in solid tumour sections by FISH is challenging to interpret^{16 17} and guidelines dealing with key technical issues and reading strategies like those available for non-small-cell lung cancer¹⁸ are not available for CRC. Thus, the lack of standardisation of analytical methods and scoring systems may partly explain why the *EGFR* GCN testing as a predictive biomarker has not been incorporated into the clinical practice yet.

We therefore designed this international ring study in order to assess interlaboratory consensus in *EGFR* copy number enumeration among five highly experienced molecular diagnostic centres with the aim of establishing variability in scoring and identifying issues that may contribute to discordant results.

MATERIAL AND METHODS

Study design

A slide-exchange programme was used to compare *EGFR* GCN FISH testing results among five pathology reference centres located in Belgium

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(University Hospital Gasthuisberg, Leuven, Belgium), Italy (Ospedale Niguarda Ca' Granda, Milano and Istituto Clinico Humanitas, Rozzano, Italy), Switzerland (Laboratory of Molecular Diagnostic, Istituto Cantonale di Patologia, Locarno, Switzerland) and USA (University of Colorado School of Medicine, Aurora, Colorado, USA). The study included testing rounds on a set of 12 colorectal cancer specimens and was coordinated by one of the participating institutions (Ospedale Niguarda Ca' Granda) where tumour specimens were selected and sent to other participating institutions; results were then analysed by two independent biostatisticians (SF and VT) (figure 1).

Specimen selection and logistics

Tumour samples were selected by the coordinating laboratory from anonymised CRC surgical specimens in such way to represent different FISH patterns. All samples were fixed with 10% neutral buffered formalin (12–48 h) and embedded into paraffin blocks. Tissue sections (4 µm thick) were mounted on positively charged glass slides with similar orientation and 25 slides were prepared from each of the 12 tumour specimens selected. Five slides from each specimen were then sent to each of the other four testing centres in a blinded manner, while five remaining slides from each specimen were retained by the sending laboratory for its own evaluation. Sequential slides were numbered following the scheme: laboratory A, slides 1, 6, 11, 16, 21 (the last one stained with H&E for morphology), laboratory B, slides 2, 7, 12, 17, 22 and so on for the other three laboratories.

Specimen analysis

FISH assays were performed by each testing centre using the EGFR/CEP7 FISH Probe Kit (Abbott Molecular, Des Plaines, Illinois, USA), according to its own operating protocol as previously described.^{7 19–21} The equipment used in each laboratory was the following: ZEISS Z 1 microscope with high-

resolution camera and full Metasystem software (Ospedale Niguarda Ca' Granda, Milano, Italy), ZEISS Axioplan 2 microscope with Metasystems CCD camera and ISIS software from Metasystems (University Hospital Gasthuisberg, Leuven, Belgium), Olympus BX 61 microscope with high-resolution camera and Applied Imaging CytoVision Genus software (Istituto Clinico Humanitas, Rozzano, Italy), ZEISS Axioskop 2 plus microscope with high-resolution camera and AxioVision software from ZEISS (Istituto Cantonale di Patologia, Locarno, Switzerland) and ZEISS AxioImager Z1 microscope with CCD camera and CytoVision/Genus software from Leica Microsystems (University of Colorado School of Medicine, Aurora, Colorado, USA). Analysis was performed according to guidelines commonly agreed by laboratory directors followed by distribution of written instructions including details on how to (1) assess quality of specimen for analyses, (2) select eight tumour foci per specimen, (3) select nuclei for scoring, (4) count the signals in each nucleus and (5) define gene amplification (online supplementary document 1). Analyses in individual cells were reported in electronic worksheets for each individual specimen and subsequently sent to statisticians for analysis. FISH scores were based on counting of *EGFR* and *CEP7* signals measured as: (1) mean *EGFR* signals per nucleus, (2) mean *EGFR*/mean *CEP7* ratio, (3) mean of percentage of cells displaying ≤ 2 *EGFR* signals, (4) mean of percentage of cells displaying ≥ 3 *EGFR* signals and (5) mean of percentage of cells displaying ≥ 4 *EGFR* signals. An additional testing was performed by one of participating centres (University Hospital Gasthuisberg, Leuven, Belgium) analysing intralaboratory reproducibility of FISH assay among different slides of the same specimen.

Statistical analysis

The reproducibility among laboratories was quantified using the standard error of measurement (SEM), which is the SD of the values within a specimen. If all laboratories assign the same value to a patient, the SEM equals 0. A SEM equal to 0.5 implies that for a specific patient, 95% of the obtained values (from various laboratories) are expected to fall in a range of $\pm 1.96 \times 0.5$ around the true value $\approx [-1; +1]$. Furthermore, the differences in values between two laboratories are expected to fall in the range $\pm 1.96 \times \sqrt{0.5^2 + 0.5^2}$, $\approx [-1.39; +1.39]$. The SEM also has been expressed relative to the mean of the values, which is known as the within-subject (specimen) coefficient of variation (WSCV).²² These indices reflect different sources of variability, that is, differences between laboratories (and/or observers), differences between slides within a specimen and measurement error. In the between-slides variability performed on the same specimen, the SEM and WSCV reflect only between-slide variability and measurement error. Mean *EGFR*/nucleus signals have been also categorised according to the following cut-offs: (1) from 0 to ≤ 2 , (2) between 2 and ≤ 3 and (3) ≥ 3 . The interlaboratory agreement for this categorisation was assessed using a (weighted) κ coefficient for multiple raters.

RESULTS

Analysis of FISH concordance among testing centres

As depicted in figures 2 and 3, scoring of mean *EGFR* GCN per nucleus (defining absolute *EGFR* GCN of a given sample) and *EGFR*/CEP7 ratio (defining *EGFR* status relative to the number of copies of chromosome 7 centromere and discriminating disomy, aneusomy or amplification) showed a low level of consensus among centres for both parameters. For mean *EGFR* gene per nucleus, SEM was 0.865, thus indicating, with a mean value of 3.22, a WSCV of 26.8%. This denotes that if these

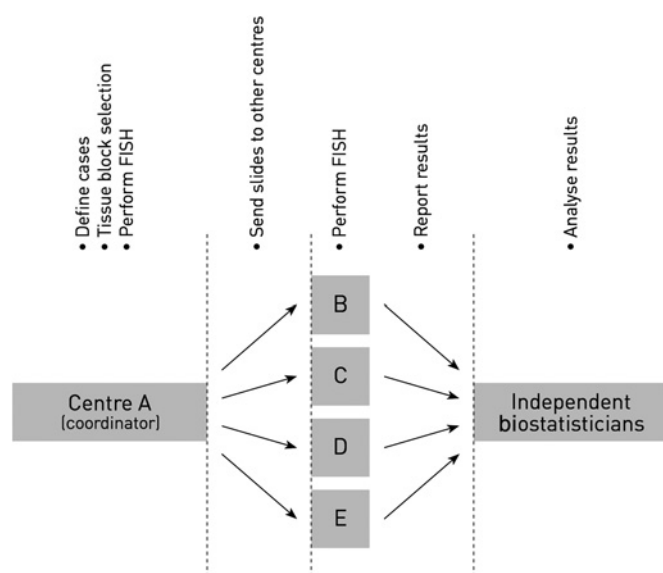


Figure 1 Workflow of the ring study. The slide-exchange programme scheduled testing rounds in which tumour specimens were selected and sent by centre A (coordinator) to centres B, C, D and E in a blinded manner; after returning to centre A, results were subjected to statistical analysis performed by two independent biostatisticians. FISH, fluorescence in situ hybridisation.

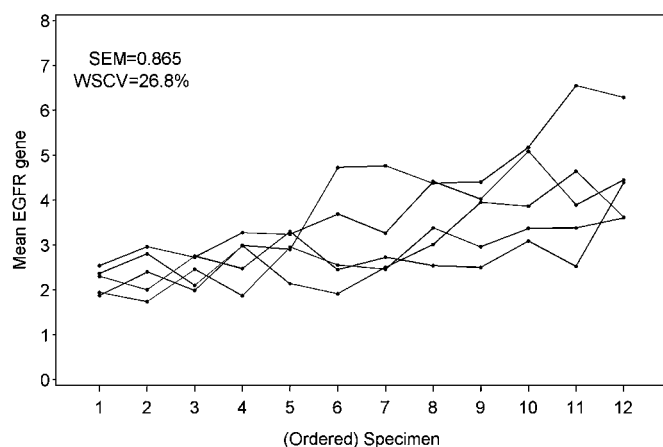


Figure 2 Reproducibility of scoring of mean epidermal growth factor receptor (*EGFR*) gene per nuclei (defining absolute *EGFR* gene copy number of a given sample) among the five institutions involved in the study. Each line represents the scorings of one laboratory given for each of the 12 specimens. The specimens are ordered according to their mean score. SEM, standard error of measurement (=within patient SD); WSCV, within-subject coefficient of variation, that is, the SEM expressed relatively to the mean value.

different laboratories evaluate a given patient, 95% of the measurements would be expected to fall in the range between values being 52.5% ($=1.96 \times 26.8\%$) lower and 52.5% higher than the true value. As an example, for a patient with a true value of 3.22, the 95% range would be between 1.53 and 4.92. For the mean *EGFR*/CEP7 ratio, the WSCV was 19.4% (SEM=0.235). A trend towards having higher variability was noticeable in specimens with higher *EGFR* copy number.

Since it was proposed that *EGFR* GCN could be proficiently scored not only as mean *EGFR* GCN/nucleus but also in terms of fraction of chromosome 7 polysomy within the tumour specimen,^{15 23} we additionally elected to test consensus among

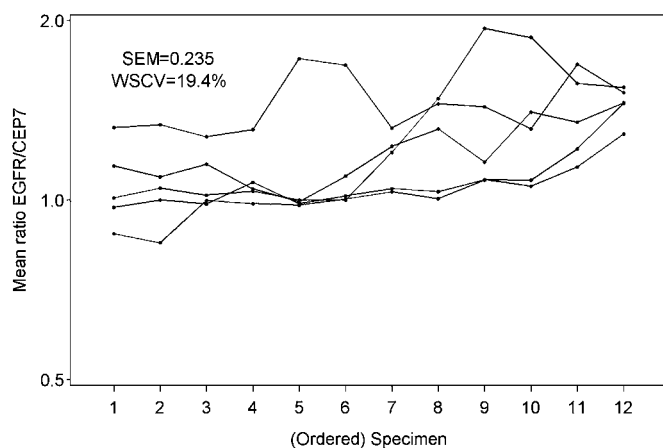


Figure 3 Reproducibility of scoring of the mean ratio of epidermal growth factor receptor (*EGFR*) gene and CEP7 (defining *EGFR* status relative to the number of chromosome 7 centromeres and discriminating among disomy, aneusomy or amplification) among the five institutions involved in the study. Each line represents the scorings of one laboratory given for each of the 12 specimens. The specimens are ordered according to their mean score. SEM, standard error of measurement (=within patient SD). To represent the ratios appropriately, the y-axis is on a logarithmic (base 2) scale. WSCV, within-subject coefficient of variation, that is, the SEM expressed relatively to the mean value.

laboratories according to the *EGFR*/CEP7 ratio. Similarly to mean *EGFR* GCN/nucleus, this parameter was associated with high variation, showing WPCV of 46.6%, 34.0% and 51.0% for percentage of cells displaying ≤ 2 , ≥ 3 and ≥ 4 *EGFR* signals, respectively (figure 4).

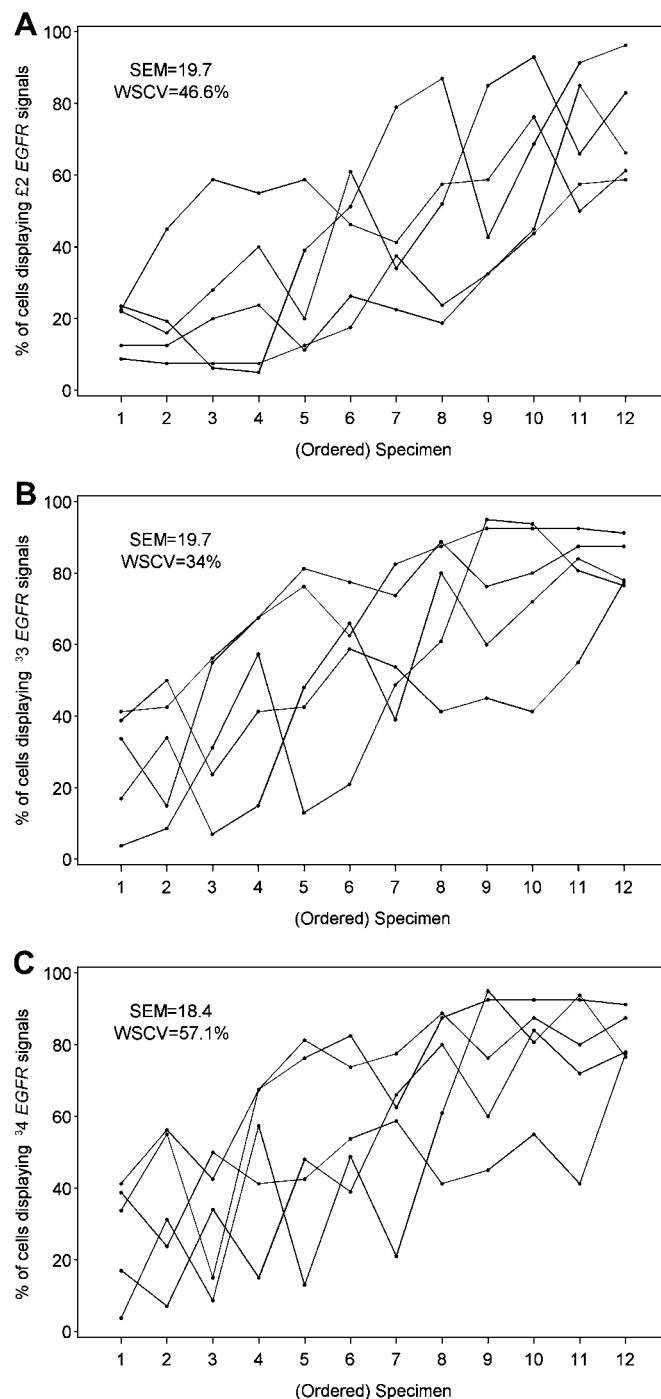


Figure 4 Scoring of epidermal growth factor receptor (*EGFR*) gene copy number was also performed according to the fraction of chromosome 7 polysomy within tumour specimens, adopting a cut-off of percentage of cells displaying ≤ 2 signals (A), ≥ 3 signals (B) or ≥ 4 signals (C). As in previous graphics, each line represents the scorings of one laboratory given for each of the 12 specimens. The specimens are ordered according to their mean score. SEM, SE of measurement (=within patient SD). WSCV, within-subject coefficient of variation, that is the SEM expressed relatively to the mean value.

Finally, consensus analysis was supported by a non-parametric approach, by ordering data of mean *EGFR* gene per nuclei according to the following cut-offs for *EGFR* GCN per nuclei: (1) from 0 to ≤ 2 , (2) between 2 and ≤ 3 and (3) >3 (table 1). In accordance with previous results, category ratings analysis indicated a low level of agreement among laboratories ($\kappa=0.202$, $SE=0.072$).

Analysis of FISH concordance within tumour specimens

Given the potential tumour heterogeneity of *EGFR* GCN within samples,²⁴ an additional testing was performed analysing intralaboratory reproducibility of FISH assay among different slides of the same specimen. As depicted in figure 5, the WSCV was 6.1% for mean *EGFR* gene per nuclei and 3.9% for mean of *EGFR*/CEP7 ratios, hence accounting only for a small fraction of the observed interlaboratory disagreement. Evaluation of fraction of chromosome 7 polysomy within the same tumour specimen resulted in WSCV of 10.5%, 12.4% and 24.0% for the % of cells displaying ≤ 2 , ≥ 3 and ≥ 4 *EGFR* signals, respectively (data not shown).

DISCUSSION

EGFR GCN has been proposed from several studies as a candidate biomarker for predicting response of CRC to anti-*EGFR* therapy by discriminating among *KRAS* wild-type patients those better candidates to cetuximab or panitumumab, thus enhancing patients' selection.¹¹ Nevertheless, these data come from retrospective analyses of patients' cohorts and there is not a reference technique for scoring. *EGFR* gene status deregulation due to true amplification, defined as more than a doubling of the *EGFR* gene compared with the CEP7 copy number, rarely occurs in CRCs.^{14 24 25} Therefore, correlation with response has been mainly based on an increase in *EGFR* gene dosage caused by

Table 1 Distribution of category ratings (top) and relative frequencies (bottom) of mean *EGFR* gene copy number in participating laboratories according the following cut-offs: (1) from 0 to ≤ 2 , (2) between 2 and ≤ 3 and (3) ≥ 3 ($\kappa=0.20168$, $SE=0.072$)

Ratings*					
Patient	Colorado	Rozzano	Locarno	Leuven	Milano
1	3	3	3	3	3
2	3	2	3	3	3
3	3	3	2	2	2
4	3	2	3	2	2
5	3	2	3	1	2
6	3	3	3	3	3
7	3	2	2	2	1
8	3	2	3	3	3
9	3	2	3	3	2
10	2	2	2	1	1
11	2	1	2	2	1
12	2	2	2	1	2
Frequencies					
Rating	Colorado (%)	Rozzano (%)	Locarno (%)	Leuven (%)	Milano (%)
1	0	1	0	3	3
	0	8.3	0	25.0	25.0
2	3	8	5	4	5
	25.0	66.7	41.7	33.3	41.7
3	9	3	7	5	4
	75.0	25.0	58.3	41.7	33.3

*Rating, categories: (1) 0 to ≤ 2 , (2) 2 to ≤ 3 and (3) >3 .
EGFR, epidermal growth factor receptor.

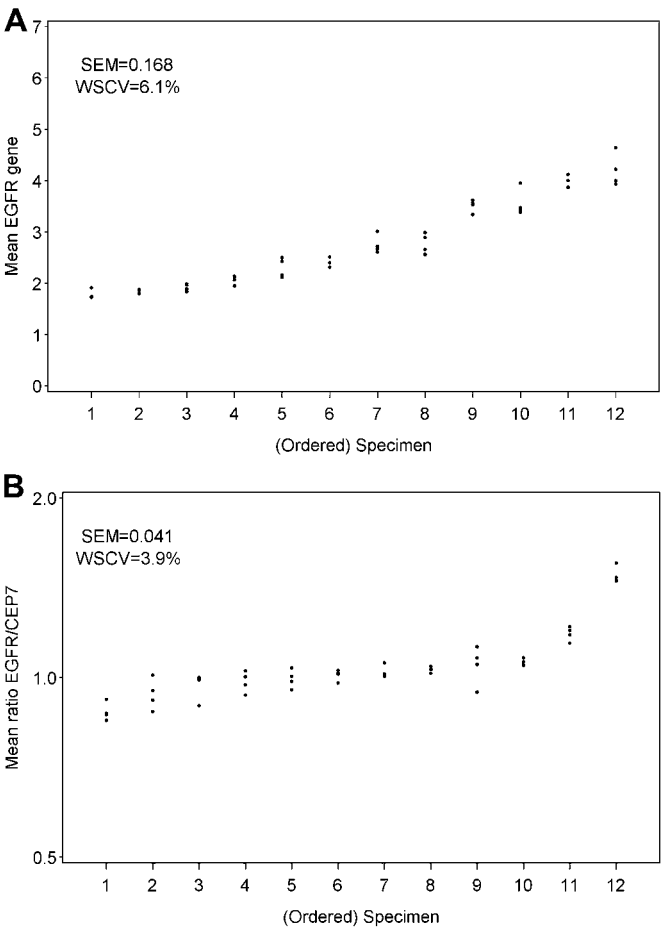


Figure 5 Intra-laboratory reproducibility of fluorescence in situ hybridisation assay among different slides of the same specimen evaluating mean epidermal growth factor receptor (*EGFR*) gene per nuclei (A) and mean ratio of *EGFR*/CEP7 (B). Each dot represents the scorings obtained on one slide for each of the 12 specimens. The specimens are ordered according to their mean score. SEM, standard error of measurement (=within slide SD). To represent the ratios appropriately, the y-axis is on a logarithmic (base 2) scale. WSCV = within sample coefficient of variation, that is the SEM expressed relatively to the mean value.

chromosome 7 polysomy, even though it is unknown whether balanced polysomy could have an equivalent biologic effect as compared with gene amplification in driving cancer progression and thus predicting response to *EGFR*-targeted agents. Discrepant cut-offs, in the range of 2.5–2.92 copies per cell, were proposed for discriminating responders from non-responders.^{20 21 23} Results of this slide-exchange ring study show that, even under standardised conditions by means of shared written guidelines and among highly experienced pathology centres, there was a low level of consensus for enumerating *EGFR* copy number in FISH assays in mCRC and that the observed variability in scoring translates into fluctuations alongside the whole range of marker usefulness. Subcategorisation of disomy (figure 4), which was recently reported to be associated with lack of response to cetuximab,¹⁴ did not improve reproducibility in our study. The study also suggests that discordant data are not due to tumour heterogeneity within samples but that the major factors for the lack of consensus should be technical, like the quality of the slide, the equipment used for the analyses and the personnel difference in interpretation of the guidelines. In this study, guidelines were discussed

and agreed upon by the laboratory directors and implemented, but a preliminary exchange of a training set of slides for refining interpretation could have helped in improving reproducibility. We assumed indeed 'a priori' that all laboratories involved in the study had the same experience and would have had similar interpretation of the guidelines, but there are many relevant skills necessary to perform enumeration of GCN in solid tumours, including identification of tumour versus non-tumour cells, focus across full depth of the section to account for complete nuclear area and, very important in CRC sections, identification of the correct individual nucleus since the individual cells may be diffusely overlapped. From this study, we learnt that in order to reach reproducible levels for all the variables explored, this strategy was not enough and that enumeration of GCN by FISH requires more intensive training to be reproducible among laboratories. For these reasons, a preliminary test of a subset of tumours according to the drafted guidelines in a process of 'familiarisation' would help by confirming that involved laboratories are doing hybridisation at the same quality level and applying same interpretation to the guidelines. The use of familiarisation for testing *EGFR* by FISH in non-small-cell lung cancer does indeed support this alternative strategy (Marileila Varela-Garcia, personal communication).

Various reports applied alternative assays for determination of *EGFR* copy number gain in mCRC, such as chromogenic in situ hybridisation,^{12–26} silver in situ hybridisation (SISH)²⁷ and quantitative real-time PCR.^{15–28} Chromogenic in situ hybridisation and SISH, if scored manually, will have at least the same limitations of FISH, although enabling simultaneous morphological tissue characterisation which could facilitate interpretation. Quantitative PCR also has limitations, both due to the poor quality of material extracted from formalin-fixed paraffin-embedded sections and the fact that genomic regions used for control may be involved in losses or gains therefore impairing the estimative of the level of gain for the test gene. Furthermore, tumour DNA dilution by healthy cells during DNA extraction can affect results of this assay. Methods to improve GCN enumeration could therefore include automation of scoring by means of software enhancing statistical power that are already available for FISH and SISH. However, automated software analysis does not work well for colorectal histology because of the irregular shape of colon cancer cells, the admixture with surrounding stroma and the need to distinguish malignant cells from normal colonic cells. These systems work better indeed for tumours with large tumour nests and for assays based on a ratio between two probes (such as *HER2/CEP17* in breast cancer) than for pure enumeration of GCN since the ratio is not affected by correctly identifying the contours of individual cells. Finally, since there is a poor correlation between *EGFR* IHC and GCN,^{17–26–29} further studies addressing whether in situ hybridisation techniques could be effectively guided by preliminary or synchronous IHC—and thus performed in areas with high *EGFR* IHC staining—should be warranted.

In conclusion, implementation of a comprehensive programme for standardisation of protocol and guidelines for *EGFR* GCN detection in mCRC is warranted in order to enhance reproducibility and subsequent dissemination into the clinic as a biomarker for predicting outcome to *EGFR*-targeted therapies. Such programme should be performed by setting up a larger follow-up study to address more in depth each parameter taken into consideration in present study in a larger sample size. A uniform protocol should be used since current results already show that existing literature cut-offs cannot be implemented and the programme should deliver not only written protocols

Take-home messages

- ▶ Even though *EGFR* GCN analysis by FISH was confirmed to have predictive value in retrospective, mostly single-institutional, series of metastatic colorectal cancer patients treated with *EGFR*-targeted therapeutics, data from this ring study show that, even under standardised conditions, molecular diagnoses of GCN could vary largely among laboratories.
- ▶ Definition of a detailed scoring system and implementation of a comprehensive training programme for molecular diagnostic laboratories are therefore necessary before including the test into clinical practice as a standard assay for predicting outcome to *EGFR*-targeted therapies.

and instructions but also technical training, possibly coupled by a familiarisation preliminary phase. Periodical proficiency testing should also be required from the clinical laboratories offering the test as a predictive assay for selecting patients.

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Contributors Conception and design of the study: AS-B, SV, MM, MF, FC, VM, AS, MG, ST, MV-G and SS. Data analysis and interpretation: AS-B, SF, NP, VT, SVB, MS, SV, MM, MF, FC, VM, AS, MG, ST, MV-G and SS. Manuscript writing: AS-B, SF, SV, MM, NP, ST, MV-G and SS. Provision of study material: AS-B, SV, MG and SS. Financial support: SS. The manuscript was approved by all authors.

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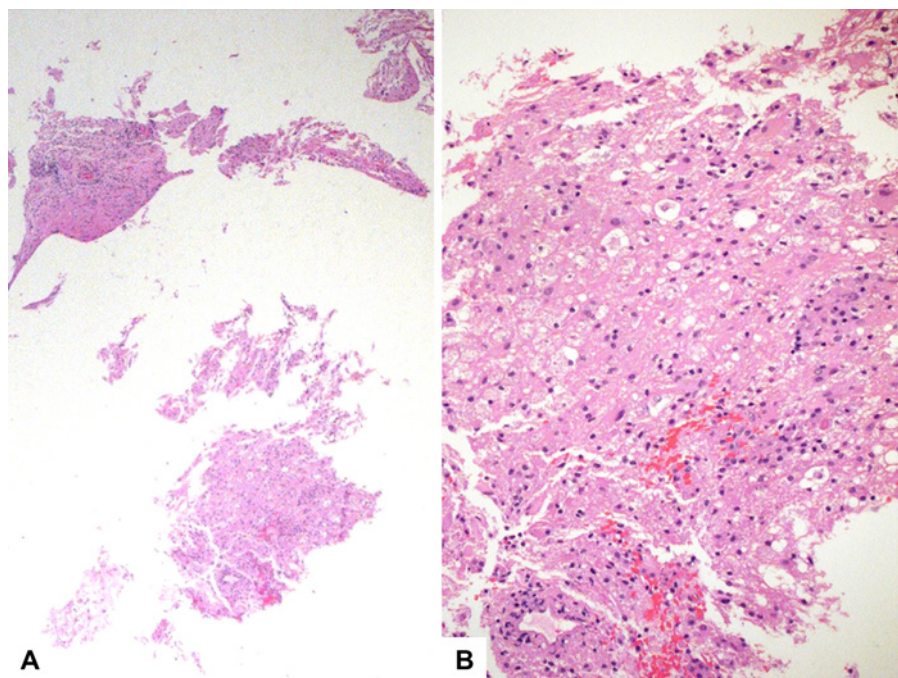


Figure 3 (A) An example of aspirated material from a cystic brain lesion, clinicoradiologically suspected to be a neoplasm, prepared into a cell block using the method as described. (B) The yield from the cystic material is ample, with excellent cytomorphological preservation, allowing a diagnosis of an inflammatory non-neoplastic lesion.

- by conventional cell blocks regardless of immediate adequacy evaluation. *Diagn Cytopathol* 2008;**36**:523–9.
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CORRECTION

doi:10.1136/jclinpath-2011-200353corr1

A Sartore-Bianchi, S Fieuws, S Veronese *et al.* Standardisation of EGFR FISH in colorectal cancer: results of an international interlaboratory reproducibility ring study. *J Clin Pathol* 2012;**65**:218–23. Figure 4 of this paper was published with errors that were made during the production process. The following information has been corrected. The y axes of Part A, Part B and Part C of the figure should be as follows: Part A: % of cells displaying ≤ 2 EGFR signals; Part B: % of cells displaying ≥ 3 EGFR signals; Part C: % of cells displaying ≥ 4 EGFR signals. The correct caption should read: Scoring of epidermal growth factor receptor (EGFR) gene copy number was also performed according to the fraction of chromosome 7 polysomy within tumour specimens, adopting a cut-off of percentage of cells displaying ≤ 2 signals (A), ≥ 3 signals (B) or ≥ 4 signals (C). As in previous graphics, each line represents the scorings of one laboratory given for each of the 12 specimens. The specimens are ordered according to their mean score. SEM, SE of measurement (=within patient SD). WSCV, within-subject coefficient of variation, that is the SEM expressed relatively to the mean value.

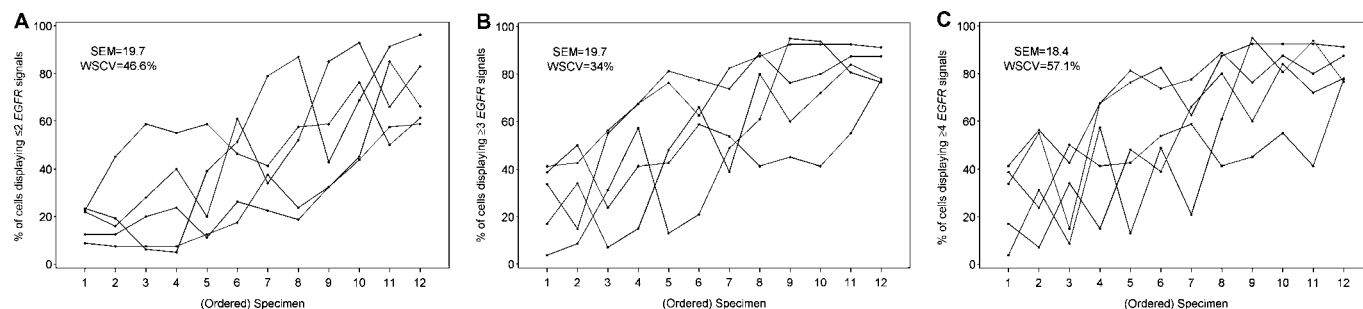


Figure 4 Scoring of epidermal growth factor receptor (EGFR) gene copy number was also performed according to the fraction of chromosome 7 polysomy within tumour specimens, adopting a cut-off of percentage of cells displaying ≤ 2 signals (A), ≥ 3 signals (B) or ≥ 4 signals (C). As in previous graphics, each line represents the scorings of one laboratory given for each of the 12 specimens. The specimens are ordered according to their mean score. SEM, SE of measurement (=within patient SD). WSCV, within-subject coefficient of variation, that is the SEM expressed relatively to the mean value.

The EGFR FISH Assay in Metastatic Colorectal Carcinoma: A Practical Guide for Analysis and Interpretation

Goal:

Standardization and Reproducibility of EGFR FISH assay among laboratories

Aspects to be Addressed

I. Specimen Preparation

II. Assay conditions and Instruments

III. Criteria for Microscope Analysis

- Quality Assessment

- Selection of Tumor Foci

- Selection of Nuclei to Score

- Imaging for Permanent Record

IV. Signal Enumeration and Recording

- Counting Signals

- Defining Gene Amplification

V. Definition of FISH pattern and Reporting

I. Specimen Preparation

Formalin-fixed, paraffin-embedded tissue according to guidelines proposed for HER2 FISH in breast carcinomas

- 1. Time from tissue acquisition to fixation:** as short as possible
- 2. Tissue fixation:** 6-48 hours in 10% neutral buffered formalin
- 3. Storage of fixed tissue:** Protected from light at room temperature
- 4. Thickness of sections:** 4 μ m +/- 1 μ m
- 5. Mounting:** on charged (coated) glass slides, sample attached ~ 1/3 distal to the frosted edge, all slides from each specimen with similar orientation
- 6. Section age:** Preferentially freshly cut (<3 months), maintained protected from light at room temperature
- 7. Material requested by the FISH laboratory**
 - a. One H-E stained and 2 unstained sections,
 - b. Preferentially from the diagnostic specimen.
 - c. Tumor content confirmed before transfer to FISH laboratory

II. Assay Conditions and Instruments

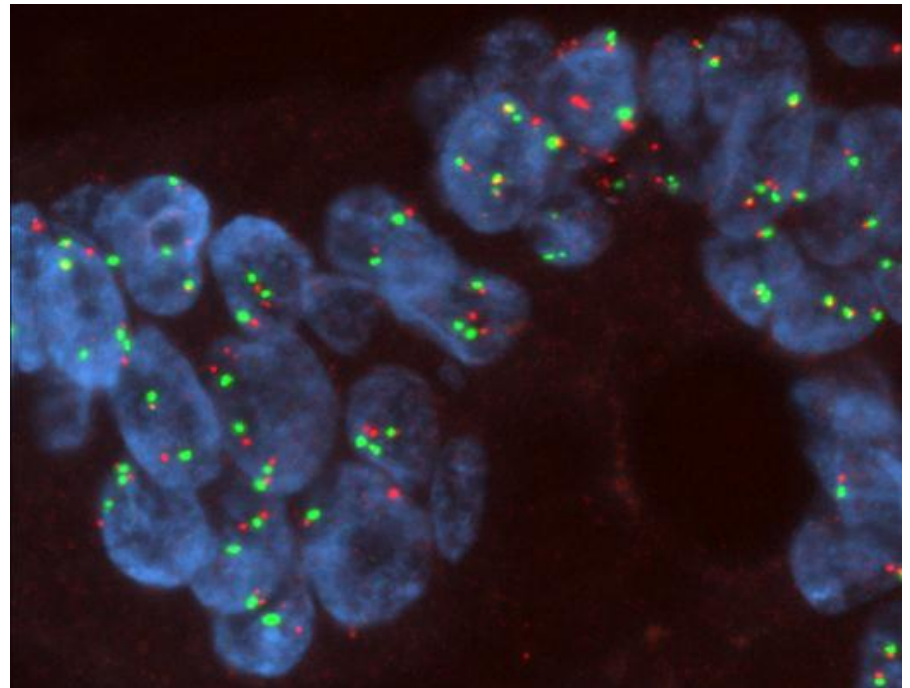
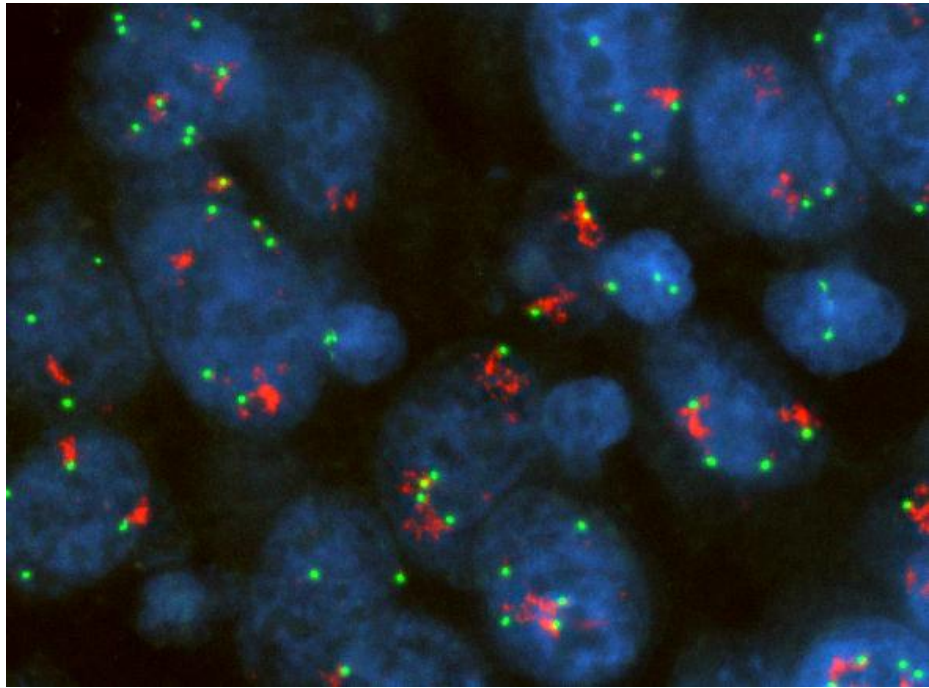
- 1. Probe:** LSI EGFR SpectrumOrange/CEP7 SpectrumGreen (Abbott Molecular)
- 2. Protocol:** Flexible as soon as quality of the results is excellent
- 3. Microscope features:**
 - a. Epifluorescence
 - b. Equipped with single band pass filters (TR, FITC, DAPI). Double and triple band pass filters highly desirable
 - c. Objectives with high NA
- 4. Imaging features:** CCD camera and Z-stacking capability

III. Criteria for Microscope Analyses

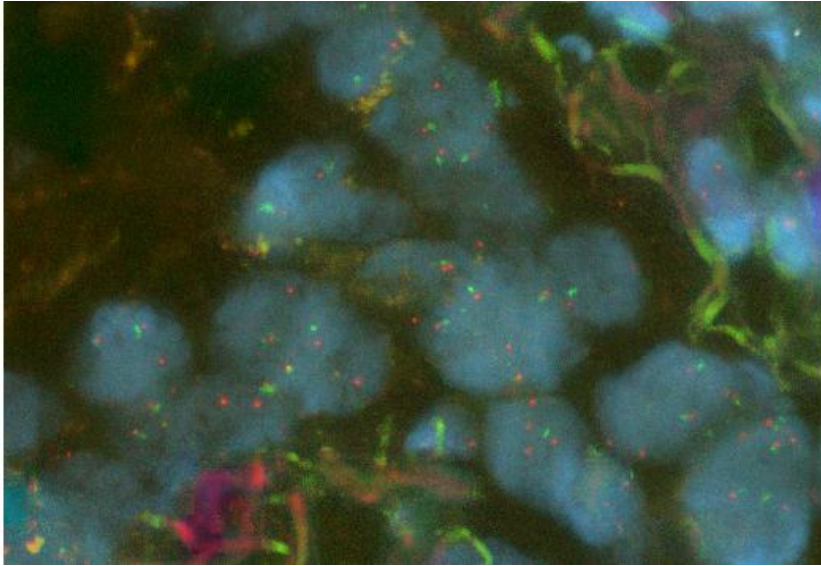
A. Quality Assessment of the FISH Specimen

1. Assess the **adequacy of the specimen for analysis** according to the requisites listed below, using high power objectives (40x, 63x or 100x). All requisites must be reached in **at least 70% of tumor cells**, otherwise specimen is classified as unsatisfactory and troubleshooting must be performed.
 - a. **Use the DAPI filter to verify integrity of the tumor nuclear morphology.** In adequate specimens, chromatin in the tumor cells is not over-treated to the point of preventing clear identification of nuclear border and is not missing from nuclear areas, and tumor nuclei are not covered by a cloudy yellowish layer or obscured by autofluorescent structures.
 - b. **Use single and double pass interference filters to inspect quality of the hybridization** in tumor and non-tumor cells (stroma reactive cells, etc). In adequate specimens, green signals (CEP 7) are bright, compact (occasionally slightly stringy or diffuse), oval shapes and red (EGFR) signals are bright, small round shapes, commonly adjacent to CEP 7 signals. The CEP 7 signal is larger and brighter than the EGFR red signal. Signals may not be fuzzy or very patchy.
 - c. **Use single and double pass interference filters to inspect the background.** It should appear dark and free of fluorescence particles or haziness.

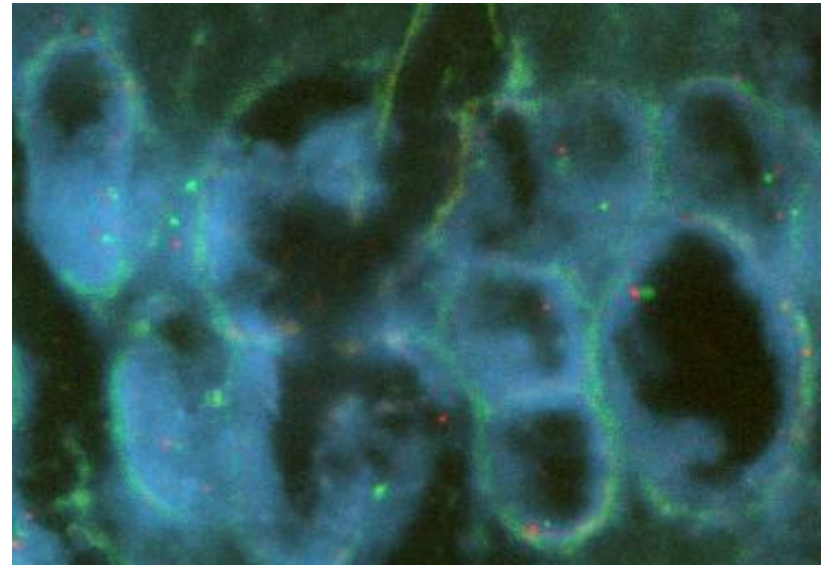
Adequate Specimen - Passed Quality Assessment



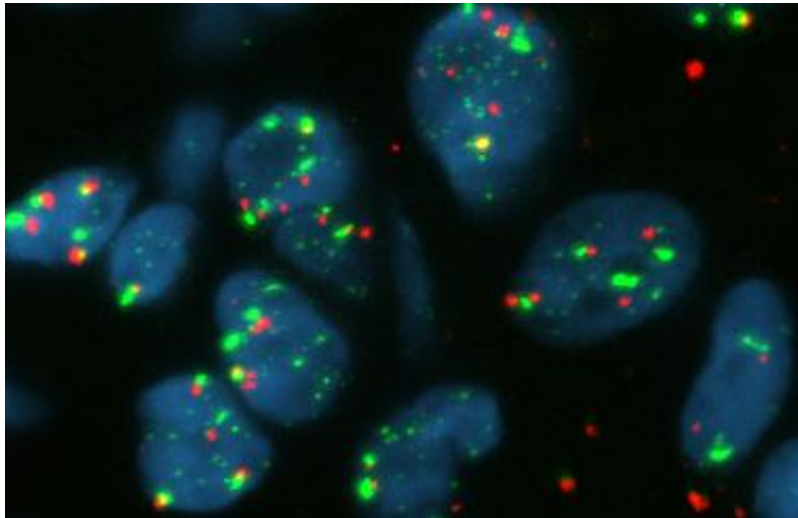
Inadequate Specimens: Quality Assessment Failure



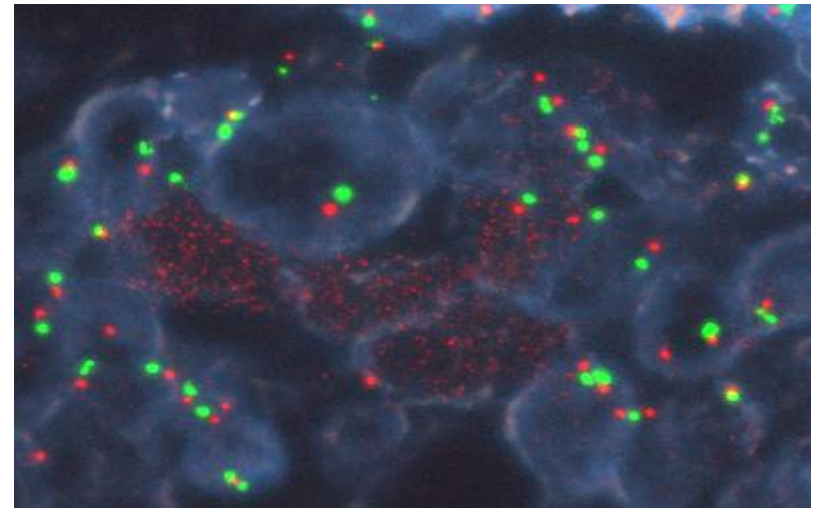
Poor signal intensity; background haziness



Missing chromatin



Cross-hybridization of CEP 7 signals



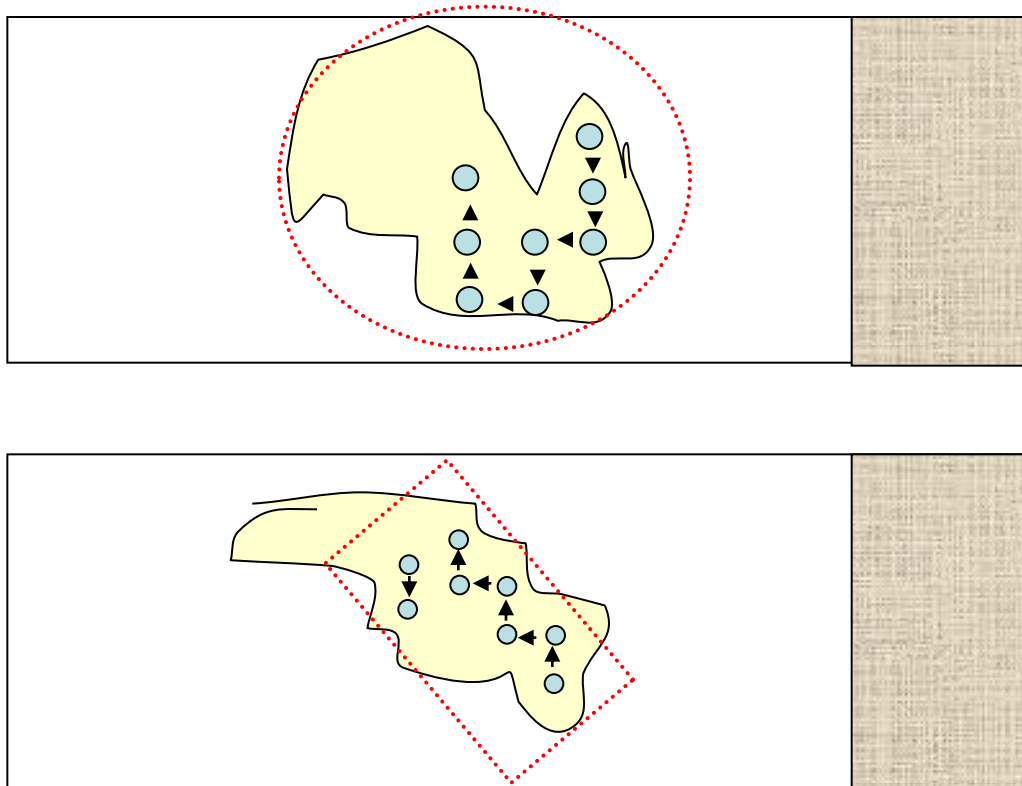
Patchy red background

III. Criteria for Microscope Analyses

B. Selection of Tumor Foci: 3 mm strategy for Heterogeneous Tumors

1. Review grossly the H&E and FISH slides searching for areas with tumor material. Select a “starting corner” (for instance, NE), identify a distinct tumor area, switch to the 100x objective and analyze approximately 10 representative cells.
2. Insert back the 40x objective, move the stage 3mm along the Y (north or south) axis, and in this 2nd location select approximately 10 cells for analysis.
3. To reach the 3rd location, repeat the sequence of steps above. After the 3rd reading in the same axis.
4. For the next 3 locations (4th to 6th), move again the stage 3 mm along the Y axis (north or south) axis.
5. Choose the next 2 locations in different X axis locations, following same schema described, up to complete the 8 tumor areas and 80 tumor cells scored.
6. When the 3 mm strategy leads to a non-tumor area do the following:
 - a. Scan the new field ± 1 mm (X or Y) to find a scorable area and resume the previous schema from that point on.
 - b. If there is not tumor area close, return to the previous location and attempt moving 3mm in a different X or Y direction.
 - c. If no sufficient tumor area is found within 3mm of the previously analyzed location, move to a completely different analyzable section of the specimen, score approximately 10 cells and then proceed with the 3mm strategy again.
7. In small biopsies or specimens with small tumor areas, the 3 mm strategy may have to be reduced to either a 1 mm or 2 mm strategy. If a different strategy has been used for analysis, it must be noted in the comments section of the analysis worksheet.

B. Selection of Tumor Foci: 3 mm Strategy for Heterogeneous Tumors



III. Criteria for Microscope Analyses

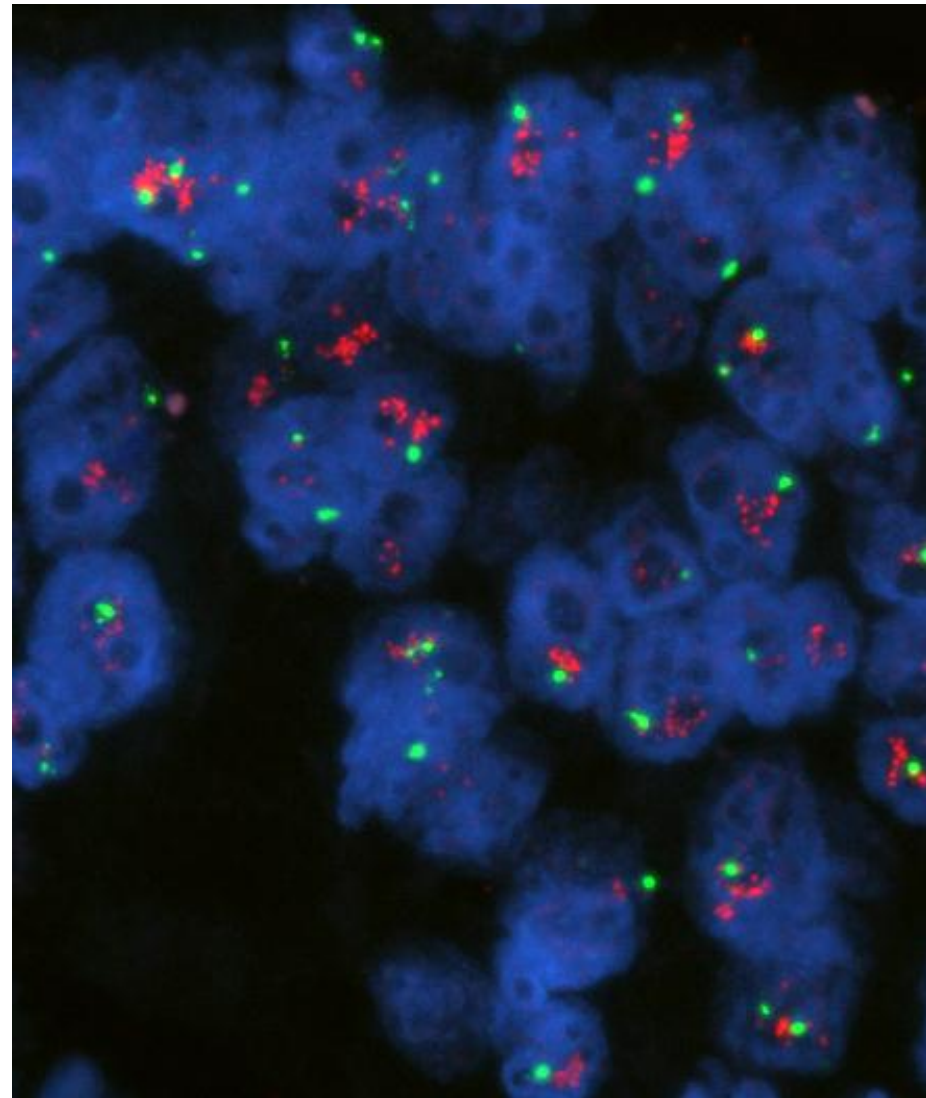
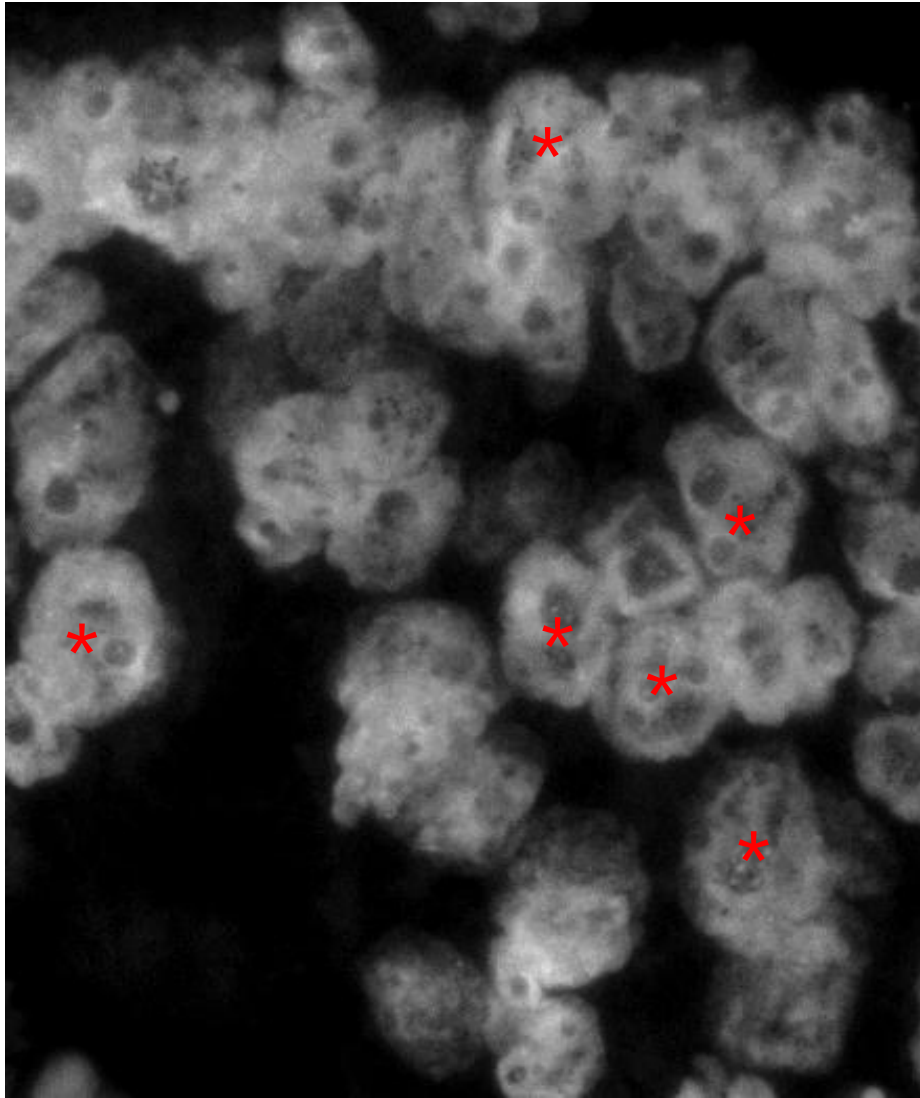
C. Selection of Nuclei to Score

1. **Select approximately 10 tumor nuclei for analysis in 2-3 microscope fields, in each of the 8 selected tumor areas.**
 - a. **Select nuclei to be scored using the DAPI filter.** Choose only nuclei that shows:
 1. Median to large diameter in comparison with other tumor nuclei in the specimen (to reduce effect of the nuclear truncation).
 2. Unambiguous borders (no overlappings, disruptions, etc).
 - b) **Confirm selection using the single or dual band pass filters.** Verify that nuclei shows:
 1. Objectively interpretable signals.
 2. At least one signal for each target.

D. Imaging for Permanent Record

1. Document results capturing at least one image of each of the 8 areas using Z-stacking .
2. Annotate the location of the imaged fields using microscope coordinates or reference slide.
3. Image any and all atypical findings not addressed in these guidelines.

Selection of Nuclei to Score



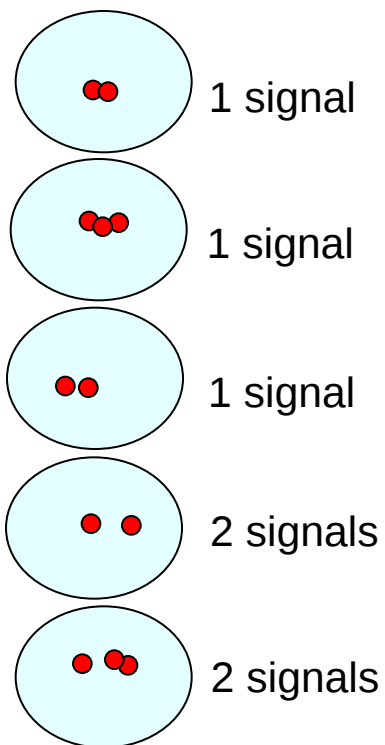
* = selected nuclei

IV. Signal Enumeration and Recording

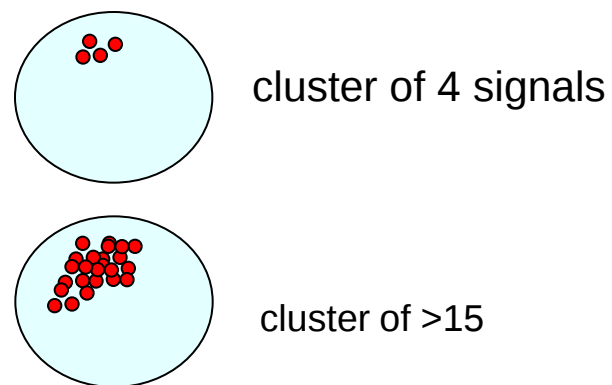
A. Counting Signals

1. Determine and record the number of EGFR (red) and CEP 7 (green) signals for each individual nucleus in the FISH analysis worksheet. Count signals according to the instructions below:
 - a) Use **single red filter to enumerate red** signals and **single green filter to enumerate green** signals. Use dual red/green and triple red/green/blue filters to verify signal numbers.
 - b) Scan the **focus through the entire depth** of the section to ensure that all signals are identified within each nucleus.
 - c) Count as 1 signal any **doublets or triplets** that are physically connected (touching, linked by a tread) or adjacent (gap smaller than the diameter of the largest signal).
 - d) Count as separate entities signals that are adjacent but separated by at least the diameter of the largest signal.
2. When nuclei do not exhibit clusters of signals, analyze at least **80 nuclei** per specimen. Report the assay as uninformative if the required number of nuclei is not available for analysis.
3. If clusters of the EGFR signal are present, proceed to (IV. B).

Counting Signals



Examples of clusters:



IV. Signal Enumeration and Recording

B. Defining Gene Amplification

1. Verify if consistently one or more copies of the **EGFR signal is atypically large** in tumor cells (brighter and larger than the CEP 7 signal) while showing the expected size in the non-tumor cells. Identify nuclei carrying this feature on the worksheet.
2. Verify presence of **clusters of signals for one or both probes**. If cells exhibit clusters of EGFR signals proceed as follows:
 - a) if **clusters are small (4 -10 copies of signals)** or very tight and enumerable, follow instructions provided in IV.A.1 and IV.A.2.
 - b) if **clusters are large (>10 copies)**, count the signals in only 30 nuclei. Enumerate as many signals as possible. If more than 15 copies are present, annotate “16” in the worksheet.
 - c) if clusters are present only in some cells or some tumor areas, identify nuclei carrying this feature on the records.

V. Definition of FISH Pattern and Reporting

1. Calculate for each specimen:

- a. % of cells showing ≤ 2 , 3, ≥ 4 copies of the EGFR signal
- b. mean copy number per cell of EGFR signals and of CEP 7 signals
- c. ratio of the mean EGFR by mean CEP 7 signals
- d. % of cells with clusters of the EGFR gene with ≥ 4 signals or atypically large EGFR signals.