FISH ON HISTOLOGICAL SECTIONS OF SOLID TUMORS: E.C.A. RECOMMENDATIONS

Test - Request for a FISH test on histological sections

The FISH test is usually requested by a pathologist as a supportive tool for confirmation or differential diagnosis *(aim: diagnosis adjunct.)*. The FISH results are utilised by the Oncologist/ Surgeon for a better definition of the biological behaviour (indolent or aggressive) of the tumor *(aim: prognosis),* and to assist in determining the correct therapeutic approach.

Materials I - Strongly recommended

- The pathologist should supply hematoxylin-eosin (H&E) stained slides with tumour areas clearly marked, along with unstained corresponding slides. The marked areas are the ones to be investigated by FISH.
- Formalin Fixed Paraffin Embedded (FFPE) tissue sections (4-5 μm) must be prepared on positively charged microscope slides, at least two for each requested test.

Materials II - Critical parameters

The fixation time and the embedding process can affect the FISH analysis, e.g. prolonged fixation time may impair access of the probe to the target DNA.

- Tissue quality (delay in fixation time) leads to decay and autolysis of the tissue.
- Some fixatives such as B5, Bouin's, Zenker's, and mercury cloride are incomepatible with standard FISH protocols, and require modification (5).

Probes - General

Specificity and Sensitivity. Specificity is the percentage of all scored signals that occur at the expected chromosomal location and sensitivity is the percentage of scorable metaphase chromosomes/nuclei with the expected signal. For clinical testing, specificity of 98% and sensitivity of at least 95% is recommended.

- For commercial probes, specificity and sensitivity are provided by the manufacturer. However, each laboratory should re-check the probe specificity (see Probes IIa), and the probe sensitivity on FFPE (see Probes IIb).
- For home–brewed probes a general validation of FISH assays is required before using them in clinical practice. No standardized published protocols are available for solid tumour FISH assays; however, a systematic procedure involving four steps (familiarization, pilot study, clinical evaluation, and evaluation of precision) is recommended (2).

Probes I - Choice of probe

Interphase FISH is performed in order to detect and to quantify the presence of specific genomic targets in non-dividing cells. **FISH detects only its intended target** and may not give information about additional chromosome abnormalities. It requires some suitable strategies such as:

- Knowledge of the specific genetic aberration (target) to be investigated: fusion genes (translocation/inversion/deletion), balanced vs unbalanced translocations, numerical changes, amplification, minimal common deleted chromosome regions.
- Choosing appropriate molecular probes designed to target alphoid DNA sequences (for numerical chromosome changes) and specific genes (for structural chromosomal changes).
- Choosing between commercially available probes vs home-brewed probes.

Probes IIa – Testing specificity

Localization: it is mandatory to confirm that comercially available/home-brewed probes detect their own intended targets.

- first time: score at least 5 male normal lymphocyte metaphases, in order to confirm the localization of the probe and to exclude cross-hybridization (including loci on the Y-chromosome). Probes with significant cross hybridization to other targets are not suitable and should not be used.
- new probe batch: run in parallel to the old one.
- if the test is used for a single locus target, an internal control (an additional probe labeled in a different colour) should be included in the probe mixture. The additional probe may be localized either on the same targeted chromosome or on a different one.

Probes IIb - Testing sensitivity

Ability of the probe to detect the intended abnormality.

- use any tumour sample known to contain the abnormality or abnormalities of interest (positive control) that have been detected by cytogenetics or RT-PCR (primary tumours or cell lines).
- use any tumour sample known to contain an abnormality or abnormalities other than those of interest (negative control).

Evaluation I - Fluorescent signals

- The evaluation of FISH signals on histological specimens should be performed by **experienced operators** using a fluorescence microscope equipped with a proper set of filters, customized for the fluorochromes used.
- Multiple colour FISH assays should be evaluated using multiple bandpass filters.
- Before starting, it is necessary to assess the adequacy of the specimen for analysis with respect to:
  - quality of signal: signals should be bright, compact, and not patchy.
  - background interference: areas with high background and/or high autofluorescence should be

avoided (background should appear as dark and as free of haziness as possible).
- the efficiency of hybridization: signals should be visible in at least 90% of tumour cells.

Evaluation II – Scanning & scoring

The quality of FISH interpretation will be compromised unless all the above-mentioned requirements are satisfied. Proceed with evaluation of the sample as follows:

- Scan the slide in low power magnification looking for tumour cell areas corresponding to the marked areas in the H&E slide.
- In each selected area, score only single non-overlapping nuclei with a clear intact nuclear membrane, avoiding areas of tumour necrosis and nuclei with ambiguous borders and ignoring cells with weak signals.

Evaluation III – Analysis

- FISH on histological sections requires a cell-by-cell analysis, hence the scoring of aneuploidy and deletion is strongly influenced by **nuclear truncation**. As a result, the sensitivity of FISH for the detection of these chromosomal alterations is reduced: the greater the number of nuclei that is evaluated the greater the confidence in detecting abnormal nuclei.
- The analysis of **fifty nuclei** may be suitable for neoplasms that are not expected to show genetic heterogeneity and in which a large percentage of the sample is expected to contain tumour cells (e.g. soft tissue tumours sarcoma).
- The analysis of **one hundred** nuclei may be desirable for neoplasms known to exhibit genetic heterogeneity or in which neoplastic cells may be focally present. **A concomitant evaluation done by a cytogeneticist and a pathologist is strongly recommended.**
- The optimal approach is to compare the analytic evaluation obtained by **two different readers**, a third reader is necessary in case of discrepancy. In doubtful cases, **a concomitant evaluation done by a cytogeneticist and a pathologist is recommended**, especially if the comparison of FISH results between marked (tumour) area(s) and non marked (morphologically normal tissue) area(s) is desired.
- Representative images of nuclei with abnormal signal patterns must be archived. An automated scoring system is not currently sufficient to replace manual analysis.

Evaluation IV – Cut-off threshold

- The presence of a particular cytogenetic abnormality should be assessed **by evaluating the frequency of a specific pattern of fluorescence signals in single nuclei and comparing it to the specific cut-off threshold value**, possibly calculated in corresponding normal tissue.
- The cut-off value is established by analyzing **a panel of histological sections of normal healthy tissue** that is used as a reference. In this context, setting up a **normal database for the specific probe for each lab, for each type of target tissue and, for the thickness of samples** (the same thickness should be maintained for all specimen testing) is strongly recommended.
- For tumours for which the normal tissue counterpart is not available or autopsy material fails to hybridize, the use of **tumour tissue of the same origin bearing a different abnormality is acceptable**.
- **All possible alternative** signal patterns should be taken into account.
- There are currently several methods to calculate the cut-off, the binomial distribution being theoretically correct and the most used.
- Limit of Detection (LoD) is also used: calculation of the upper limit of the abnormal signal pattern in normal cells.

Abnormalities - Loss and gain, amplification, detection of gene disruption

Losses and gains are detected by a dual/multiple colour FISH assay using probes specific for the chromosome region or gene of interest and, as a control, an alphoid/centromeric probe or a single gene probe located on the same chromosome.

Aneuploidy is usually detected by centromeric probes specific for individual chromosomes, labelled with different fluorochromes.

Deletion / allelic loss is detected using a dual colour FISH assay with a probe specific for the region of interest and, as a control, a probe that is localized on the same chromosome but not involved in the deletion.

Gene Duplications / multiple copies / amplifications.

- The gene of interest is considered to be duplicated when the number of its FISH signals exceeds the number of FISH signals of the control probe by one.
- The gene of interest is considered to be present in multiple copies as a result of polysomy when the number of FISH signals of the gene of interest is equal to the number of signals of the control probe. A gene is considered to be amplified when the number of FISH signals of the gene of interest exceeds the number of FISH signals of the control probe by more than two. Very often the FISH signals of the gene of interest occur as a cluster.
- NOTE. It should be noted that when using commercially available HER-2 tests for breast cancer, the guidelines for the interpretation of amplification recommended by the manufacturers should be followed (a ratio >2.2 is usually indicated). The international pathology community is trying to

improve the status of HER2 testing in routine practice (2,6,7).

Gene disruption caused by chromosomal rearrangements leading to fusion genes can be detected with dual colour FISH using various strategies: a) dual colour break-apart or split-signal, b) dual-colour dual-fusion and rarely, c) dual-colour single fusion.

- a) ***dual colour break***-apart or split-signal strategy: two differently labeled molecular probes flanking, or partially involving, the gene under investigation, are used. If there is a break in the gene, as a result of a translocation or an inversion, the two differently coloured signals will appear split apart. The distance between the split signals depends on the design of the break-apart probe, which must be known and taken into consideration in the evaluation (e.g. a gene broken because of a translocation will usually show two split signals further apart than those split by an inversion). The homologue that is not involved in the translocation will be represented by the two signals close to each other. With this method the rearrangement in the gene in question is detected but the partner region involved in translocation remains unknown. This method is therefore useful for identifying rearrangements which may involve different partner chromosomes or where the partner gene is unknown.
- b) **dual-colour dual-fusion** strategy: two differently labeled molecular probes are hybridized to two different genes on two chromosomes, covering all possible breakpoint regions. A reciprocal translocation involving the two genes would result in two fusion signals. The homologues not involved in the translocation will be represented by two differently coloured signals. This method is sensitive and useful for detecting specific chromosomal translocations.
- c) **dual-colour single fusion** strategy: two differently labeled molecular probes that do not include all the breakpoint regions are hybridized to the two chromosomes involved in the translocation. One probe maps to the proximal end of the breakpoint in one chromosome and the other one to the distal end of the breakpoint in the other chromosome. As a result of the translocation the probe from one chromosome moves next to the other probe on the other chromosome creating a fusion signal. The homologues not involved in the translocation will be represented by two differently coloured signals. This method is rarely used, and is useful for detecting unbalanced translocations.

Reporting results – Important points

Results of FISH on histological samples should be reported according to the national rules. It is however recommended that the FISH report contains:

- **FISH findings** described according to the latest ISCN recommendations (8).
- **name** and **manufacturer of the probe**, or the **BAC name** for the home-brewed probes.
- **simple statements**: normal, abnormal, inconclusive.
- laboratory cut-off value for each probe. Care should be taken when the **test value falls around the cut-off value and when reporting negative results**.
- **paraffin block identification number** (selected by the pathologist and indicated in the request form) from which the slide for FISH investigation was prepared.
- **any disclaimer** according to national regulation for genetic testing laboratories in each country.
- **signature: according to the rules of each country**. Ideally, either the cytogeneticist signs the cytogenetic report and the FISH findings are included in the final pathology report or the pathologist and the cytogeneticist co-sign the final report.

References

1. European Cytogeneticists Association (E.C.A.). Permanent Working Group for Cancer cytogenetics, solid tumor studies. Interphase FISH on solid tumours: recommenddations for use on histological sections in daily practice. Newsletter 2011, No. 27: 5-11.
2. Wiktor AE, Van Dyke DL, Stupca PJ, Ketterling RP, Thorland EC, Shearer BM, Fink SR, Stockero KJ, Majorowicz JR, Dewald GW. Preclinical validation of fluorescence in situ hybridization assays for clinical practice. Genet Med. 2006. 8:16-23.
3. Weremowicz S, Schofield DE .Preparation of cells from formalin-fixed, paraffin-embedded tissue for use in fluorescence in situ hybridization (FISH) experiments. Curr Protoc Hum Genet. 2007; Chapter 8:Unit 8.8.
4. Wolff AC, Hammond MEH, Schwartz JN, Hagerty KL, Allred DC, Cote RJ,Dowsett M, Fitzgibbons PL, Hanna WM, Langer A, McShane LM, Paik S, Pegram MD, Perez EA, Press MF, Rhodes A, Sturgeon C, Taube SE, Tubbs R, Vance GH, van de Vijver M, Wheeler TM, Hayes DF. 2007: American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer. J Clin Oncol. 2007;25:118-45.
5. Schurter MJ, LeBrun DP, Harrison KJ. Improved technique for fluorescence in situ hybridisation analysis of isolated nuclei from archival, B5 or formalin fixed, paraffin wax embedded tissue. Mol Pathol. 2002. 55: 121-4.
6. Hsu CY, Li AF, Yang CF, Ho DM. Proposal of Modification for the Definition of Genetic Heterogeneity in HER2 Testing in Breast Cancer. Arch Pathol Lab Med. 2010;134:162.
7. Albarracin C, Edgerton ME, Gilcrease MZ, Huo L, Krishnamurthy S, Middleton LP, Resetkova E, Sahin AA, Sneige N, Fraser WS, Wu Y. Is It Too Soon to Start Reporting HER2 Genetic Heterogeneity? Arch Pathol Lab Med. 2010;134:162-63.
8. ISCN (2009): Shaffer L.G., Slovak M.L., Campbell L.J.(eds). Recommendations of the International Standing Committee on Human Cytogenetic Nomenclature, Basel: S Karger, 2009.