

Introduction

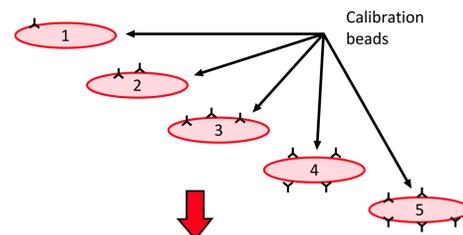
- Cell line controls in immunohistochemistry (IHC) and in situ hybridisation (ISH) have become established as a tool for monitoring assay performance. Their use has become increasingly prominent due to their application as system controls in prognostic and predictive assays, including the evaluation of HER2 status in breast cancer patients for whom Herceptin® is being considered.
- Whilst we acknowledge the application of control cell lines as analyte and external quality assurance (EQA) monitoring tools, it is important to emphasise that appropriate tissue controls, fixed and processed in the same manner as diagnostic material, remains the gold standard in laboratory assay control. The use of control cell lines provides complementary and consistent monitoring for both commercial systems and EQA.
- The aims of this update are to:
 - Outline the characterisation procedures that are implemented, to ensure that the control cell lines utilized by UKNEQAS ICC & ISH in the Breast HER2 IHC and ISH modules maintain the highest standards of consistency and reproducibility.
 - Describe how control cell lines can accurately reflect appropriate tolerances of laboratory based HER2 assays, ensuring they are a viable and constructive control device for UKNEQAS ICC & ISH participants.

Cell Line Characterisation

- Receptor load assessment of control cell lines is an important step in characterising cells lines as analyte controls. This verification step complements the subsequent protein and gene profile analysis, using the established diagnostic methodologies; IHC and FISH. Here we briefly describe the characterisation and analysis of 4 breast cancer cell lines using a flow cytometry based methodology.

Figure 1a, b and c. Cell line receptor load analysis using mouse monoclonal anti-HER2 antibody (clone CB11).

1a. Murine IgG coated calibration beads with 5 different antibody binding sites concentrations.



1b. 5-point standard curve created using calibration beads of known labelled mouse IgG binding site concentration tagged with FITC-conjugated goat anti-mouse immunoglobulins.



1c. Separate suspensions of whole cells from each breast cancer cell line were fixed, then permeabilised to allow CB11 monoclonal antibody (mouse anti-human) to bind to the internal domain of the HER2 receptor. Following incubation and washing, cells were labelled with the secondary layer, FITC-conjugated goat anti-mouse IgG. The fluorescent signal was then quantified using flow cytometry and the signal plotted on the standard or calibration curve, to enable an approximation of receptor load per cell.

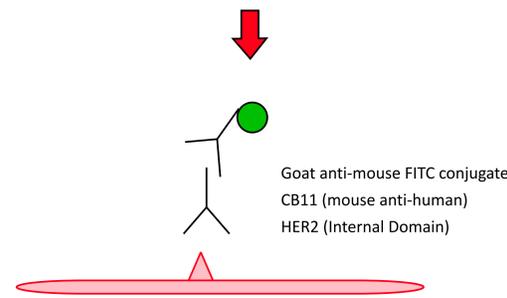


Table 1. HER2 protein profile by IHC, gene status by FISH and corresponding receptor load by flow cytometry, taken from the Oracle HER2 Bond IHC System - Instructions for Use, Leica Biosystems.

Cell Line	HER2 IHC Profile ^A	HER2 Receptor Load per Cell [*]	HER2 Gene Status ⁺	
			HER2 Copy Number	HER2:Chr17 Gene Ratio
SK-BR-3	3+	4.3x10 ⁵	13.35	3.55
MDA-MB-453	2+	1.4x10 ⁵	5.73	2.05
MDA-MB-175	1+	6.3x10 ⁴	3.33	1.20
MDA-MB-231	0	9.3x10 ³	3.15	1.13

HER2 protein profile^A as assessed by IHC (Oracle HER2 Bond IHC System)

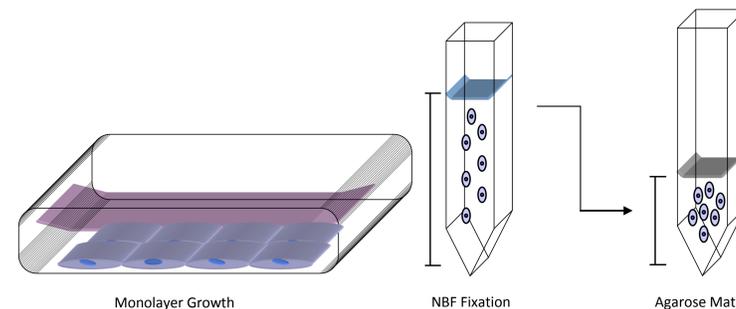
HER2 receptor load^{*} analysis as assessed by flow cytometry (Dako Qif)

HER2 gene status⁺ as assessed by dual probe FISH (Abbott Molecular PathVysion HER2 DNA Probe Kit)

Growth of Cell Lines

- Many commercially available cell lines are adherent in culture and grow as a monolayer. Key features of this growth process include:
 - Providing nutrients and an environment tailored to the requirements of each cell line.
 - Limiting repeated passage of cells and using cells within a passage number window as close as possible to the passage number of the initial culture.
 - Harvesting cells mechanically as opposed to chemically.
- Optimisation of the above processes (see Figure 2) enables the generation of control material which accurately reflects cell morphology and protein expression.
- Long-term observation also enables monitoring of:
 - Recognition of a sudden genetic mutation that may result in altered HER2 gene/protein expression.
 - Increases confidence in the ability to supply control material of required specification.
 - Possible expansion of defined selection criteria windows through generation of increasing amounts of experimental data.

Figure 2. Illustrating monolayer cell growth, fixation in suspension, through to capture in an agarose matrix.



Cell Line Fixation & Processing

- Following appropriate harvesting, cells are fixed in 10% (4%w/v) neutral buffered formalin (NBF). NBF is also the recommended fixative for breast cancer cases which are to be submitted for HER2 evaluation^{1,2}.
- Although cell line protein expression will always show some degree of cell to cell variation within a single harvest, due to individual cellular expression phases, fixation of cells in suspension enables the cells to fix in a relatively homogeneous manner, allowing for a more uniform staining profile. Captured in an agarose matrix, the cells are then processed through graded alcohols, cleared in xylene through to paraffin wax.

References

1. Wolff A C, Hammond E H, Schwartz J N, Hagerty K L, Allred D C, Cote R J, Dowsett M, Fitzgibbons P L, Hanna W M, Langer A, McShane L M, Palk S, Pegram M D, Perez E A, Press M F, Rhodes A, Sturgeon C, Taube S E, Tubbs R, Vance G H, van de Vijver M, Wheeler T M & Hayes D F. American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer. Journal of Clinical Oncology, Vol 25, No1, 2007.

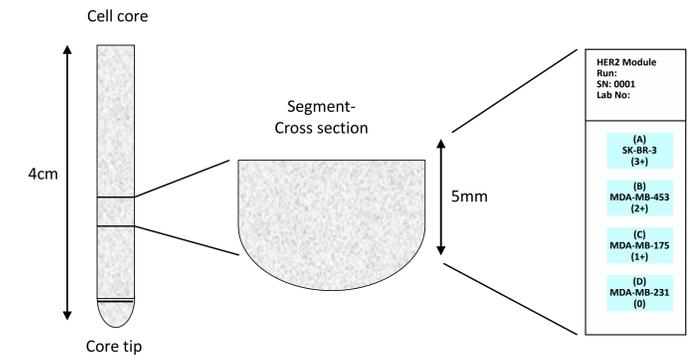
2. Walker R A, Bartlett J, Dowsett M, Ellis I O, Hanby A, Jasani, B, Miller K, Pinder S. HER2 Testing in the UK – a further update to recommendations. Journal of Clinical Pathology. doi:10.1136/jcp.2007.054866; published online 1 Apr 2008.

3. Barker C, Ibrahim M, Miller K & Reid V. Non-destructive quality control of HER2 control cell line sections: the use of interferometry for measuring section thickness and implications for HER2 interpretation on breast tissue. Applied Immunohistochemistry & Molecular Morphology. Accepted for publication 27 Feb 2009.

Embedding, Orientation & Section Preparation

- Fixed and processed cells are embedded in polymer based embedding wax and are sectioned at 3µm and dried overnight at 37°C followed by 1 hour at 60°C to facilitate adherence for harsher antigen retrieval methodologies (e.g. pressure cooker). Cut sections are orientated as shown below, however the order of the cell lines may be varied from EQA run to run.

Figure 3. Uniform cell distribution and layout of the UKNEQAS control cell lines.

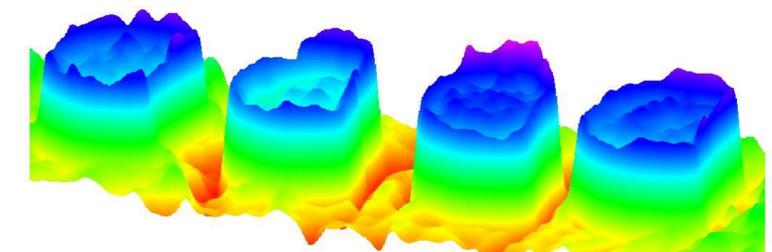


- Cutting sections at 3µm facilitates single cell adherence to charged slides, ensuring that there is minimal carryover between adjacent cell spots through complete single cell adherence to the charged surface. The presence of un-adhered cells migrating between cell spots is a key characteristic of sporadic, thick cell line sections. An awareness of section thickness is crucial when assessing control cell lines and in certain cases may be a cause of false positive/negative results, participants and assessors should be mindful of this when staining cell lines for assessment. Precise 3µm sectioning gives a more delineated, sharper IHC stain.

Section Thickness Measurement (Interferometry)

- Based on work published by Barker *et al* 2009, each UKNEQAS IHC and ISH HER2 cell line control slide is non-destructively tested using a Leica Biosystems patented white light interferometry system³.
- This technique measures specifically the thickness of the MDA-MB-453 cell line core, based on a measurement from the surface of the glass slide to the top of the paraffin section and is used to screen out all sections above 3.6µm in thickness. Barker *et al* established the 3.6µm cut off point as the measurement in which the observed perception of the equivocal (2+) HER2 grade starts to increase in intensity.
- Furthermore, increased section thickness of the cell line preparation can result in carryover from un-adhered multilayered cells as reagent flows over the section, potentially resulting in carryover between cell spots. Non-destructive section thickness measurement ensures that the preparation remains as a monolayer of cells, which is fully adhered to the glass slide.

Figure 4. Topographical image of the UKNEQAS IHC and ISH HER2 control cell line spots on a glass slide as generated by white light interferometry³. This technique provides an accurate method for consistent HER2 cell line control slide manufacture that stain reproducibly.



- This unique process ensures accurate section thickness is maintained, enabling consistency in control slide staining. This level of control is critical to achieving accurate HER2 assay validation and continuous batch performance.