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Introduction: This study assesses the feasibility of using Quantitative Real-Time PCR (qRT-PCR) to resolve HER2 amplification/over-expression status in invasive breast cancer cases that fail resolution via immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) testing following ASCO/CAP guidelines. The IHC and FISH equivocal patient population (Double Equivocal) represent a particularly problematic breast cancer sub-group that currently lacks standardized clinical management guidelines. Methods: Cases were selected from the Cleveland Clinic electronic records from 1/2008 to 12/2010. RNA extraction was performed following macro-dissection using High Pure RNA Paraffin Kit (Roche Applied Biosciences, Indianapolis, IN). qRT-PCR was carried out using TaqMan® RNA-to-CT™ 1-Step Kit with primers and probes (HER2, B2M, GAPDH, ACTB, TFRC, Applied Biosystems, Foster City, CA). qRT-PCR was performed on a LightCycler 480 II (Roche Applied Biosciences, Penzberg, Germany) according to the manufacturer’s instructions. Results were expressed as the ratio of HER2 to reference gene copies, all normalized against calibrator RNA from MCF7. Results: qRT-PCR performed on two breast cancer control groups, HER2 amplified (AMP) and non-amplified (Non-AMP) as defined by FISH and IHC, demonstrated 2 non-overlapping populations. ROC curve analysis, using a cut off of 7.0, showed the qRT-PCR assay separates AMP from Non-AMP cases with 100% sensitivity and specificity. Applying the 7.0 RT-PCR cut off to a group of double equivocal cases resulted in resolution of HER2 amplification/expression status for all cases (10 AMP and 40 Non-AMP). Cases with heterogeneity of HER2 expression did not alter sensitivity of the RT-PCR assay. Conclusions: qRT-PCR analysis of HER2 gene expression represents a viable approach to resolve cases with double equivocal HER2 status at the time of diagnostic biopsy. This molecular approach accurately determined HER2 status in a population that failed classification by both FISH and IHC. qRT-PCR combines the precision and high sensitivity of real-time PCR with the morphological specificity of histological evaluation and ultimately allows definitive HER2 classification at the time of initial diagnosis. Further studies correlating response to anti-HER2 therapy and HER2 status by qRT-PCR are warranted.