Chapter 12

Quantitative, Fluorescence-Based *In Situ* Assessment of Protein Expression

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Summary

As companion diagnostics grow in prevalence and importance, the need for accurate assessment of *in situ* protein concentrations has increased. Traditional immunohistochemistry (IHC), while valuable for assessment of context of expression, is less valuable for quantification. The lack of rigorous quantitative potential of traditional IHC led to our development of an immunofluorescence-based method now commercialized as the AQUA® technology. Immunostaining of tissue samples, image acquisition, and use of AQUA software allow investigators to quickly, efficiently, and accurately measure levels of expression within user-defined subcellular or architectural compartments. IHC analyzed by AQUA shows high reproducibility and demonstrates protein measurement accuracy similar to ELISA assays. The process is largely automated, eliminating potential error, and the resultant scores are exported on a continuous scale. There are now numerous published examples where observations made with this technology are not seen by traditional methods.

Key words: Immunohistochemistry, Quantitative analysis, Biomarkers, AQUA, Tissue microarrays,

1. Introduction

Quantitative assessment of protein and RNA has been increasing in accuracy and multiplicity for years, but nearly all quantitative techniques require dissolution of the substrate and hence loss of spatial information. Immunohistochemistry can be both quantitative and maintain the spatial information. The decreased size of clinical pathology specimens and the increased need for accuracy have Progressively pushed the tissue biomarker field into quantitative immunohistochemistry. Currently, semiquantitative pathological

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scoring of markers is important in disease management decisions. Scores include one of four discrete, ordinal categories (0, 1+, 2+, and 3+) for HER-2 staining or estimated percent positivity for estrogen and progesterone receptor. Some groups use a combination of intensity and area staining as in the Hscore *(1)* or the Allred system *(2)*. However, all of these systems suffer from the problems of subjectivity, noncontinuity, and variable reproducibility. These problems and related issues have led to the first effort at standardization published recently as the ASCO/CAP guidelines for assessment of HER2 expression *(3)*. Since the results of this assay in the clinical setting determine whether or not a patient will receive a course of therapy (often costing over $100,000) *(4)*, it seems likely that this will be the first of a number of such efforts toward standardization of critical companion diagnostic assays.

Historically, quantitative assessment has been done using both chromagens and fluorescent methods. While chromagens have the advantage of providing context, through the addition of hematoxylin counterstains, they also have disadvantages. Fluorescence-based detection systems can be multiplexed and can generally provide a broader dynamic range than chromogens. Here we focus on a system developed by our lab, but now commercially available, that allows quantitative assessment of protein expression with accuracy comparable to an ELISA assay but without loss of spatial information.

AQUA® (originally an abbreviation for Automated QUantitative Analysis but now trademarked) technology uses multiple fluorescent labels on conventional formalin-fixed, paraffin-embedded histology material. Images are then captured at all appropriate wavelengths to measure expression based on the colocalization and intensity of the immunostain *(5)*. AQUA analysis of IHC staining has shown comparable accuracy to protein detection by ELISA assay *(6)* and is currently being used to quantify expression on both tissue microarrays (TMAs) *(7)* and whole sections *(8)*. TMAs have facilitated the development of AQUA since they have facilitated standardization by arraying of cell lines and multiple samples on the same slides for assessment of reproducibility. This protocol describes the use of AQUA in assessment of a TMA, but it can be similarly used for whole sections.

2. Materials

2.1. Immunohistochemical Staining of Formalin-Fixed, Paraffin-Embedded Tissue for Fluorescence Microscopy

1. Xylenes, use in hood.
2. *Citrate Buffer*. 3.84 g sodium citrate dehydrate in 2 L double deionized water, pH to 6.
3. *Pressure cooker*: 6-quart aluminum pressure cooker (Presto).
4. *Peroxidase Blocking Buffer*. 10 mL 30% H_2O_2 in 390 mL Methanol.

5. *Tris-buffered saline (10× TBS)*. For 5 L stock, 438 g NaCl, 121.8 g Trizma Base (Sigma), 4,500 mL deionized H_20, pH to 8 with concentrated HCL, bring up to 5 L with H_20).
6. *TBS wash buffer (1×TBS)*. From 10×TBS stock.
7. *TBS with Tween 20 wash/diluent buffer (TBS-T)*. TBS (1×) with 0.05% (v/v) Tween 20 (Sigma).
8. *Primary antibody dilution buffer*. 0.3% (w/v) Albumin Bovine Serum, Fraction V, ≥ 96% (Sigma) in TBS/T, (BSA/TBS-T).
9. *Primary target antibody*. For example, mouse antihuman ERα clone 1D5; (DAKO, Carpinteria, CA).
10. *Primary mask antibody*. Rabbit antibovine cytokeratin (wide-spectrum screening, WSS; Z0622, DakoCytomation, for use with mouse target primaries) or Mouse AntiHuman Cytokeratin (Clone AE1/AE3 DakoCytomation, for use with rabbit target primaries are used here for detecting breast cancer, appropriate masks should be determined for different tissue types (see discussion of masking later).
11. *Secondary target antibody*. DAKO EnVision™ + System, HRP, goat antimouse for mouse target primaries or antirabbit for rabbit against target primaries.
12. *Secondary mask antibody*. Goat antirabbit (secondary to rabbit cytokeratin) or goat antimouse (secondary to mouse cytokeratin); Alexa-546 (Invitrogen; Eugene, OR) applied in low-light conditions.
13. *Target flourophor*. Cy5-Tyramide Reagent Pack with amplification diluent (PerkinElmer; Boston MA) applied in low-light conditions.
14. *Mounting medium with nuclear stain*. Prolong Gold antifade reagent with DAPI "4′6-diamidino-2-phenylindole" (Invitrogen).
15. Dark slide chamber with moistened filter paper and supports to hold slides for humid incubation.

2.2. AQUA ® Image Acquisition and Analysis System

Image acquisition is now performed on the HistoRx PM-2000 platform (HistoRx, New Haven, CT). This platform is the professional optimization of the original platform developed in our lab around an Olympus BX51 microscope, a Cooke Sensicam High Performance camera, and a Prior Proscan automated stage control. The PM-2000 captures images from either TMAs or whole sections and provides a stack of high-resolution monochromatic images for analysis by the AQUA software. The images are viewable, but examination of the images by "eye" is not part of the scoring system.

The images are then analyzed by the AQUA program, which generates a table of AQUA scores and associated sample

coordinates. The software operational details are available from HistoRx.

3. Methods

3.1. Immunohistochemical Staining

Staining procedures listed have been optimized for use with the AQUA automated image acquisition system and analysis software. Other than changes to target antibody dilution, manipulations are rarely made. As an example, Images and data shown in this chapter are for ER-α on breast carcinomas. Adjustments for antibody dilution, incubation time and selection of antibodies for tumor mask, and compartment selection should be made according to the question of interest and tissue under study.

1. *Day 1.* Melt paraffin off slides in 60°C incubator for 20 min or until slides are translucent. Remove residual paraffin by incubating in two changes of Xylenes for 20 min each. Wash slides two times in 100% ethanol for 1 min each time, followed by one wash in 70% ethanol also for 1 min. Transfer slides to tap water with a slow gradient under running water, and transfer to 100% water for at least 5 min for rehydration. Pressure cook slides in citrate buffer for 10 min from the time lock pin slides up, indicating the unit has pressurized. Cool under running tap water for 10 min. Incubate slides in peroxidase block for 30 min. Wash with two changes of tap water for 30 s each and transfer to (1×) TBS wash buffer. Dry slides carefully around the array edge with a fresh Kimwipe, adding enough BSA/TBS-T to fully cover array area. Incubate in humidity chamber with BSA/TBS-T for 30 min at room temperature. Decant off BSA/TBS-T by tipping slide along its long edge against a paper towel, and again dry the edges with a fresh Kimwipe. Store in (1×) TBS while preparing cytokeratin antibody (1:100) in BSA/TBS-T. Add enough to each slide to cover array area and incubate overnight at 4°C. (Note: typically, primary target antibodies are applied with cytokeratin antibody and incubated overnight. For some antibodies, such as ERα/ 1D5, however, this is too long and nonspecific staining increases).
2. *Day 2.* Make primary ERα antibody solution (1:50) in BSA/TBS-T and cover all tissue on slide, incubating in humidity chamber for 1 h at room temperature. Decant off antibodies and rinse briefly with TBS-T. Wash all slides twice in (1×) TBS-T for 10 min each and once in (1×) TBS for another 10 min. Dry slides around edge with fresh Kimwipe and apply goat antirabbit Alexa-546 solution (1:100) in goat antimouse

Envision (neat) to all slides. Incubate in humidity chamber for 1 h at room temperature. Decant off secondary antibodies and repeat washing procedure as described above. Dry slides around edge with fresh Kimwipe, and apply Cy5-tyramide solution (1:50) in reagent pack amplification diluent to each slide. Wash all slides twice in TBS-T, first for 10 min, then for 5. Wash a final time in (1×) TBS for 5 min. Dry slides around edge with fresh Kimwipe, and apply Prolong Gold with DAPI (50–75 μL). Gently place cover slip over array and let dry overnight in the complete absence of light.

3. Clean surface of coverslip with ethanol if necessary, and seal around edges of the cover slip with clear nail polish to further preserve stain and prevent fading. Note: We have successfully removed coverslips applied in this manner using acetone to remove sealing polish and incubating slides in warmed phosphate-buffered solution.

3.2. Image Acquisition

Capturing images of the spots on a TMA or fields from a whole slide uses a software package that comes with the PM2000 platform for image collection. Specific instructions are included and training and support is available from the vendor (HistoRx, New Haven CT). Although the process is somewhat automated with some human intervention, the key steps are enumerated as follows.

Initially, the software acquires a series of low-resolution (4×) images, producing a scan of the entire slide using a fluorescence cube set selected by the user (often the channel marking tissue nuclei). For TMAs, signal from the low-resolution image is optimized to produce image acquisition coordinates reflecting areas of interest across the array, represented by spots, which are validated by the user. Aligning the resulting spots into corresponding rows and columns, as well as assigning the order with which image files will be created, creates a text file with saved spot coordinates. At each individual spot, the script takes an in-focus image and an additional out-of-focus image 8 μm below the original focal plane for all fluorophores selected by the user. For example, an in and out-of-focus image is taken with the blue filter (DAPI/nuclear compartment) filter before rotating to repeat with emission filters for the mask (Orange, Alexa-546) and the target protein (Red, Cy5). According to the coordinates assigned in **step 3**, the microscope continues this procedure for all spots on the array, saving all images to a chosen computer directory**.

3.3. AQUA and Expression Quantification

The underlying basic assumptions of AQUA are that any entity must be defined by a molecular interaction, rather than a contrast-generated edge (as is seen in feature extraction software). For example, nuclei are defined by colocalization with DAPI

pixels, rather than by sphericity or roundness. A second basic premise is that every concentration is composed of a numerator and a denominator. Thus to generate an *in situ* concentration, we measure the amount of signal (the sum or the intensities in the target pixels) as the numerator and divide by the area of the pixels of interest (or the user-defined compartment). We define the following terms for use of AQUA **(Fig. 1)**:

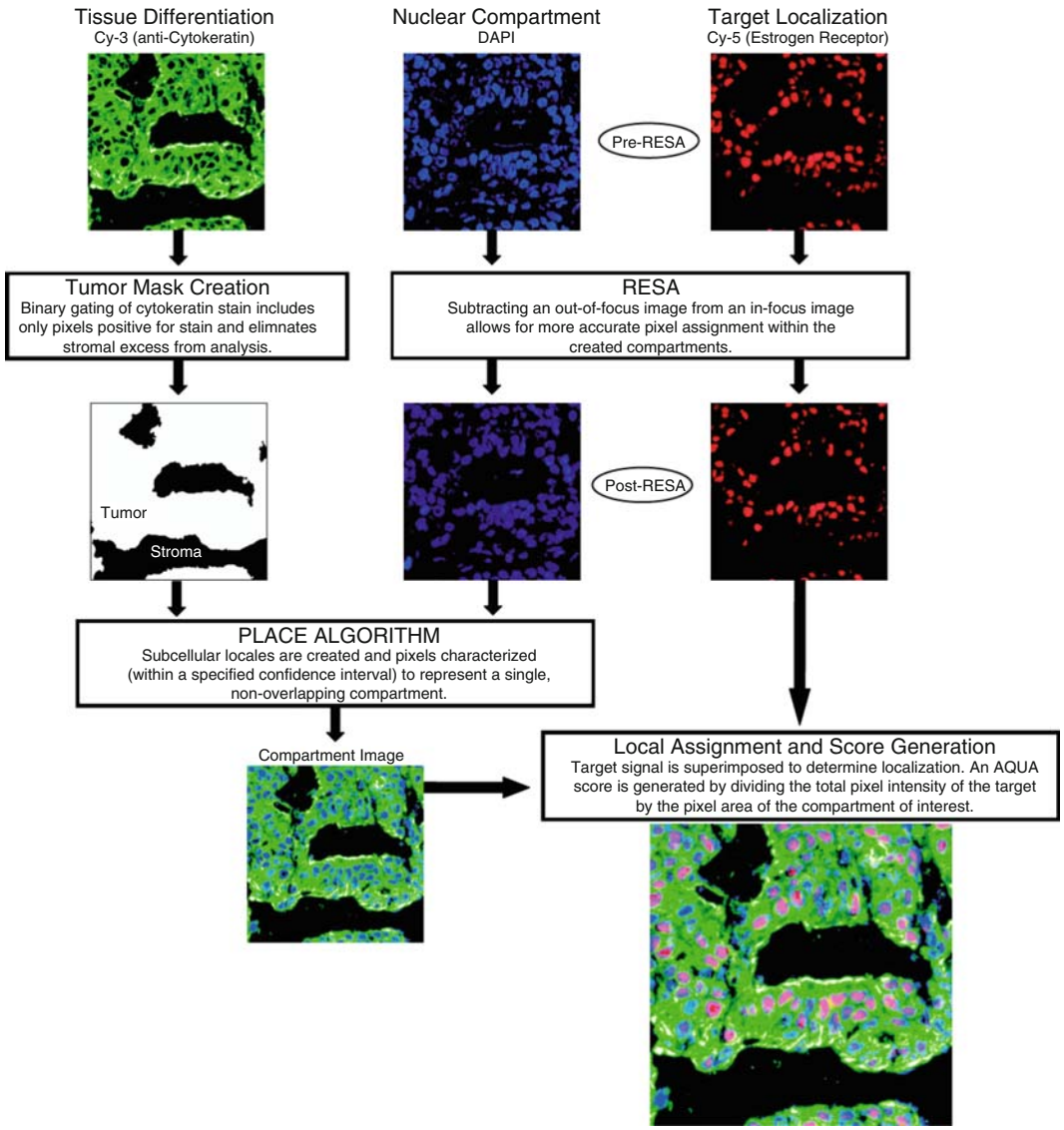

Fig. 1. An AQUA Schematic Diagram illustrates the main objectives of the AQUA software components including identifying tumor, creating subcellular compartments, and localizing/measuring target protein expression within these compartments (*See Color Plates*).

1. *Mask*. The user-defined region of interest as selected by a molecular interaction. For example for epithelial tumors, cytokeratin is used as a mask. For neural tumors, we use GFAP, etc. The goal is that the mask excludes stromal tissue or blank space.
2. *Compartment*. The compartment is also defined by a molecular interaction, but ultimately results in a set of pixels that represent the desired entity (for example a pan-cadherin antibody might be used to define a membrane compartment). To quantify tumor protein expression, the compartment pixels must be within the Mask. The definition of which pixels are within a compartment is subject to either manual or automated threshold determination, and can be assisted by the RESA process defined later. Compartments may be traditional subcellular membrane-bound structures (like nuclei or golgi) or virtual structures (like a kinase) as long as there is a molecular interaction to uniquely define a set of pixels. Compartments can also be cellular or architectural features like T-cells, vessels, or lymphatics.
3. *Target*. The target is the protein being measured. The intensity is measured and summed over all of the pixels within the defined compartment. Usually long-wavelength fluorophores (emission peaks greater than 650 nm) are used for targets to minimize interference due to autofluorescence.

With the acquired in-and out-of-focus images, AQUA software is used to generate scores (equivalent to *in situ* concentrations) for each of the user-defined targets in each of the user-defined compartments. Initially, the tumor mask is designated by the binary gating of pixels in which staining exceeds the threshold. Setting an intensity threshold assigns positively stained pixels as "on" and discards the rest as "off," followed by slight dilation and hole-filling algorithms to produce a mask which is an accurate representation of tumor area within the histospots or fields **(Fig. 1)**. For ERα, the DAPI stains of the nuclei are used in generating nuclear compartments. Subtracting the generated nuclear compartment from the tumor mask creates a non-nuclear compartment, which includes both the cytoplasm and the membrane, which is sufficient for addressing many issues that require resolution only in the 1 μm range.

Since we do not use confocal or convolution/deconvolution imaging to remove out-of-focus light, the accurate assignment of compartments requires an extra step. We take a second out-of-focus image for each filter for application of the rapid exponential subtraction algorithm (RESA). The RESA algorithm works by utilizing the out-of-focus image for each histospot or field to subtract the light in a manner inversely proportional to the intensity in the out-of-focus image. Details of the exact methods

are in US patent #7,219,016. New extensions of RESA are in development to reduce nonspecific background in all channels. The PLACE algorithm uses the remaining signal and ensures the most accurate compartmental assignment possible. Applied to the target, it assigns pixels to nonoverlapping user-created compartments within a 95% confidence interval. AQUA scores are then generated as the sum of the total pixel intensity of the target within the compartment of interest (the numerator) divided by the pixel area of that same compartment within the tumor mask (the denominator) *(5)* **(Fig. 2)**.

After all spots or fields in an array have been run through the script, intensity thresholds set, and compartments created, the final step in the quantification process includes validation of each individual spot. This includes adjusting thresholds, cropping out adjacent histospots, and refining percent of remaining values of the RESA algorithm. Data quality is improved by having trained investigators review each image to either be included in, or discarded from overall, final analysis. There is also automated validating software available from HistoRx that can review images for inclusion or exclusion. The extent and aggressiveness of the validation process varies with the specific biological question being addressed. To completely remove any subjectivity, this step is performed by the HistoRx software.

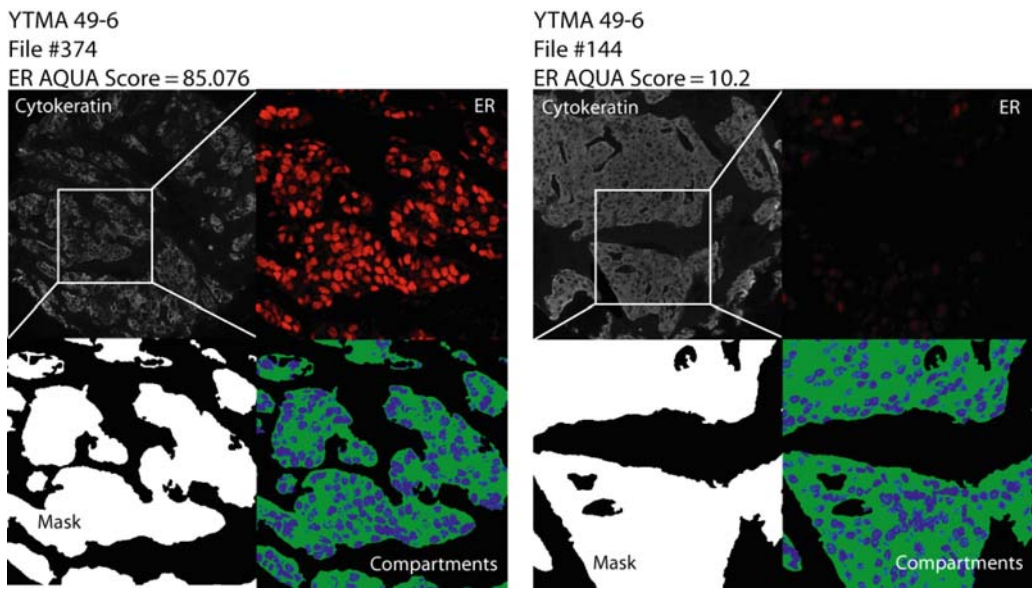

Fig. 2. Two examples of estrogen receptor expression based on AQUA analysis are shown using a set of images that illustrates the monochromatic cytokeratin image, the mask, the compartments, and the target at two magnifications so the reader can evaluate all of the features that generate the AQUA score. File #374 on the left with a nuclear compartment AQUA score of 85 is an example of a high expression level. File #144 on the right, with an AQUA score of 10.2, is an example of low expression of ER (*See Color Plates*).

3.4. Standardization and Assessment

After completion of AQUA-based analysis the results need to be assessed for reproducibility and standardization. These are key features that can show the rigor of results obtained using this method, but are very difficult to assess on conventionally stained material. Reproducibility is best assessed by reading multiple spots (or fields) from the same tumor in independent experiments. The collection of two- or three-fold information generates increased confidence in both the experimental result and the method. Many studies have been done to address the issue of the number of spots required to be representative of a whole section *(9–11)*, and most suggest between 2 and 4 depending on tumor type. Collection of at least 2 spots allows linear regression and/or evaluation of Spearman's ρ to assess the reproducibility of the assay. **Figure 3** shows an example of the reproducibility we have seen in a cohort of breast cancer cases assessed for estrogen receptor. Generally ρ values between 0.6 and 0.9 are seen in high quality, reproducible assays, where values nearer 0.6 suggest high degrees of marker heterogeneity within the tumor. Serial sections from the same TMA block typically give ρ values in the 0.95–0.98 range. When the ρ value drops below 0.5, there is usually a systematic error to be discovered and we have seen this between different lots of antibody from the same vendor *(12)*. This assessment is generally applied to TMAs, but can also be applied to fields within whole sections *(8)*.

Standardization is also important for rigorous assessment of *in situ* protein expression. To achieve standardization, we have used cell lines, prepared as tissue. By creating a series of TMA

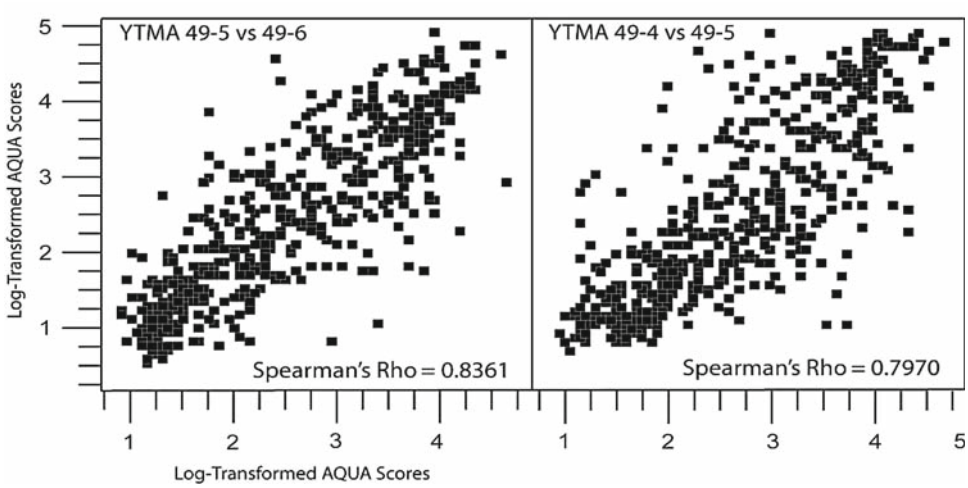

Fig. 3. Assessment of reproducibility using linear regression shows high levels of correlation with Spearman's ρ values around 0.8. These arrays contain regions of tumor cored at different times and locations for each patient, showing the level of marker heterogeneity and assay reproducibility. ρ values range from 0.6 to 0.9 depending on tumor heterogeneity. Values below 0.5 often are a sign of systematic error or low-quality antibody.

spots from cell lines, absolute values can be obtained in pg/mg total protein *(6)*. This can be done by standardizing against ELISA assays or other methods of measurement of protein within cell lines. This method can also be used to find the "biological" cutpoint for the threshold of expression. **Figure 4** shows a number of cell lines that are ER negative or ER positive, thus allowing selection of the AQUA score that is the threshold for positive for any given performance of the assay.

The method for preparation of formalin-fixed paraffin-embedded cell lines for standardization controls is as follows:

Cell preparation varies with cell lines used; however for all cell lines, immediate fixation is critical. Thus cells are grown in flasks to desired density, fixed by removing medium and then directly adding formalin to the culture flask. Cells should not be trypsinized or pretreated prior to fixation as that may generate artifacts of hypoxia or other cellular stress. Adherent cell lines can be disassociated from the flask using a cell scraper after fixation.

1. Start with a generous amount of cells (often 6–8 T150 flasks) suspended in 10% neutral buffered formalin in a 50-mL conical.

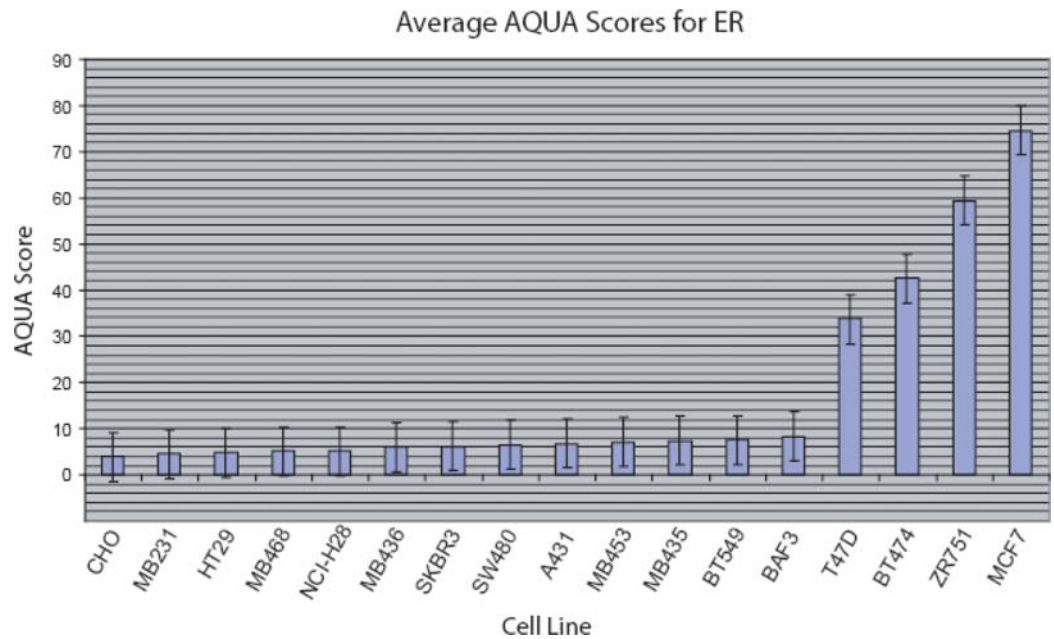

Fig. 4. This histogram shows expression levels of a number of cell lines ranked by expression level of ER. This allows visualization of a plateau of negative cases, suggesting the threshold of AQUA scores associated with system noise. When the curve breaks, levels of expression rise above background. Based on the AQUA scores of cell lines on the array, a cutpoint can be determined based on ELISA assays or other in vitro assays of the same cell lines. Even without knowing the exact concentration, graphing in this manner allows determination of a biological cutpoint for the threshold of ER expression (in this case at an AQUA score of 10).

2. Prepare 10 mL of 1% Low Melt agarose in 1× phosphate-buffered saline (PBS). Incubate for at least 1 h at 55°C to get solution to a consistent temperature.
3. Prepare 0.6-mL Eppendorf tubes, each with approximately 50 µL of paraffin (Surgipath, Blue Ribbon Tissue Embedding Infiltration Medium), liquefying paraffin in bottom of tube by incubating for 5 min at 60°C. Remove from incubator and refrigerate to harden. (This makes a flat surface at the bottom of the tube and a pellet that is easier to work with in **step 5**.)
4. Centrifuge 50-mL conicals with fixed cells at 1,200 rpm for 5 min.
5. Gently aspirate supernatant and wash with approximately 30 mL of 1× PBS. Repeat twice, resuspending in 1× PBS and centrifuging each time.
6. Remove 1× PBS from final wash and resuspend in approximately 0.4 mL of 80% ethanol. Transfer to 0.6-mL Eppendorf tube from **step 2** (or 1.5-mL tubes for large volumes of cells).
7. Pack cells by centrifugation at 12,000 rpm for 5 min.
8. Gently remove ethanol, leaving pellet of cells in the bottom. Repeat by adding 0.4 mL of 80% ethanol and centrifuging again for 5 min. (This removes the last traces of PBS and helps the cells remain stuck together).
9. Again, gently aspirate off ethanol. Using a Heyman probe or a similar small tool, transfer cell pellet to a labeled Eppendorf cap (removed from its tube with a razor blade). Mold pellet into cap with the Heyman probe.
10. If pellet does not fill the cap, use approximately 75 µL of 1% agarose (from **step 1**) to fill voids around the edges of the cap. Work quickly and put caps immediately into a freezer. Let sit overnight.
11. Pop the pellets of cap-molded cells into labeled tissue cassettes (Therm-Fisher). Store overnight submerged in 80%ethanol before taking to histology department for embedding.
12. Embed pellets in vertical position in the center of a paraffin block without eosin as eosin increases autofluorescence.

4. General Notes

1. To see a broad range of expression it is important to titer antibodies to optimal dilutions prior to image acquisition.

This issue and its implications for accurate measurement of protein expression are addressed in McCabe et al. *(6)*.

2. The most frequently altered steps of the staining protocol involve incubation time adjustments for BSA/TBS-T block and primary target antibodies. If primary target antibodies do not stain optimally at 4°C overnight, they are usually incubated at room temperature for 1–2 h.
3. Because compartmentalization is so dependent on successful stains for each of the deposited fluorophores, when a spot does not show a DAPI or Mask protein stain, it must be discarded. The strict application of validation can result in elimination of as many as 20% of the spots on an array.
4. For some tissues, it is difficult to find appropriate masking proteins. For example, melanoma is masked with anti-S100 antibody, which stains some other cells as well, that should not be considered in analysis.
5. Catenin and cadherin family proteins can be used to construct more accurate membrane compartments, as cytokeratin can only be localized with AQUA to the cortical cytoplasm. We have had the most success with alpha-catenin since it seems to be preserved in most tumors, where cadherins and beta-catenin is often reduced or lost.
6. During AQUA validation, spots with extremely small compartmental/tumor mask areas may show inaccurately high AQUA scores due to such a small denominator. Consequently, tumor mask areas less than 5% of the total histospot's area are generally excluded from analysis.

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