A microplate-based assay for analyzing purity and mispairing of bispecifics and other complex molecules in cell culture samples

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Background and novelty: We are presenting the world´s first assay for assessing mispairing and purity of complex biotherapeutics based on charge differences in a microplate format. Mispairing is a huge challenge in the production of bispecific antibodies and other complex molecules. It refers to the situation where heavy and light chains of bispecific molecules incorrectly pair with each other, producing product-related impurities that must be monitored during the bioprocess development. We illustrate the assay principle using standard monoclonal antibodies (mAbs) with different isoelectric points and show data from a stable pool screening of a bispecific biosimilar.

Experimental approach: We use the patented PAIA*plate* assay technology with cation exchange capture beads and a pH gradient. Beads and pH-gradient are provided in the ready-to-use PAIA microplate. A fluorescence labeled Fc-specific binder with high affinity enables detection of the interaction of the biomolecules with the negatively charged beads at different pH levels and low concentrations.

Results: This novel assay generates charge profiles of complex mixtures differently charged molecules. The evaluation of ternary mixtures of standard mAbs with different isoelectric points shows that the assay can distinguish these molecules. Thus, this assay can determine the content of individual species, including impurities, based on their different charge profiles. We are also showing that stable pool samples containing different levels of correctly assembled bispecific products could be analyzed and samples with high amounts of bispecific product were successfully identified.

Assay principle

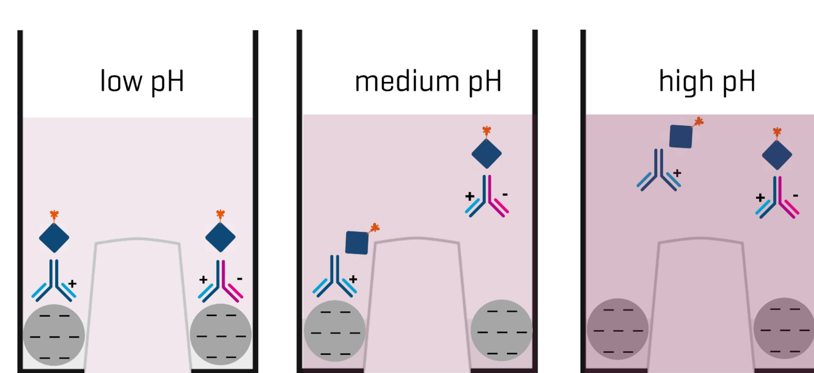

Figure 1. Assay principle of the Purity and Mispairing Assay (Art.-No PA-401)
The wells of the PAIA*plate* contain negatively charged cation exchange beads and a fluorescence labeled binder that detects the interaction of the analyte with the negatively charged cation exchange beads. The antibodies are completely bound to the beads at low pH levels (**left**) and are released at higher pH (**right**). The special design of the PAIA*plate* allows to measure this release directly after the reaction is finished and the beads have settled. More information about the PAIA technology is available at www.paiabio.com.

Assay data generation and interpretation

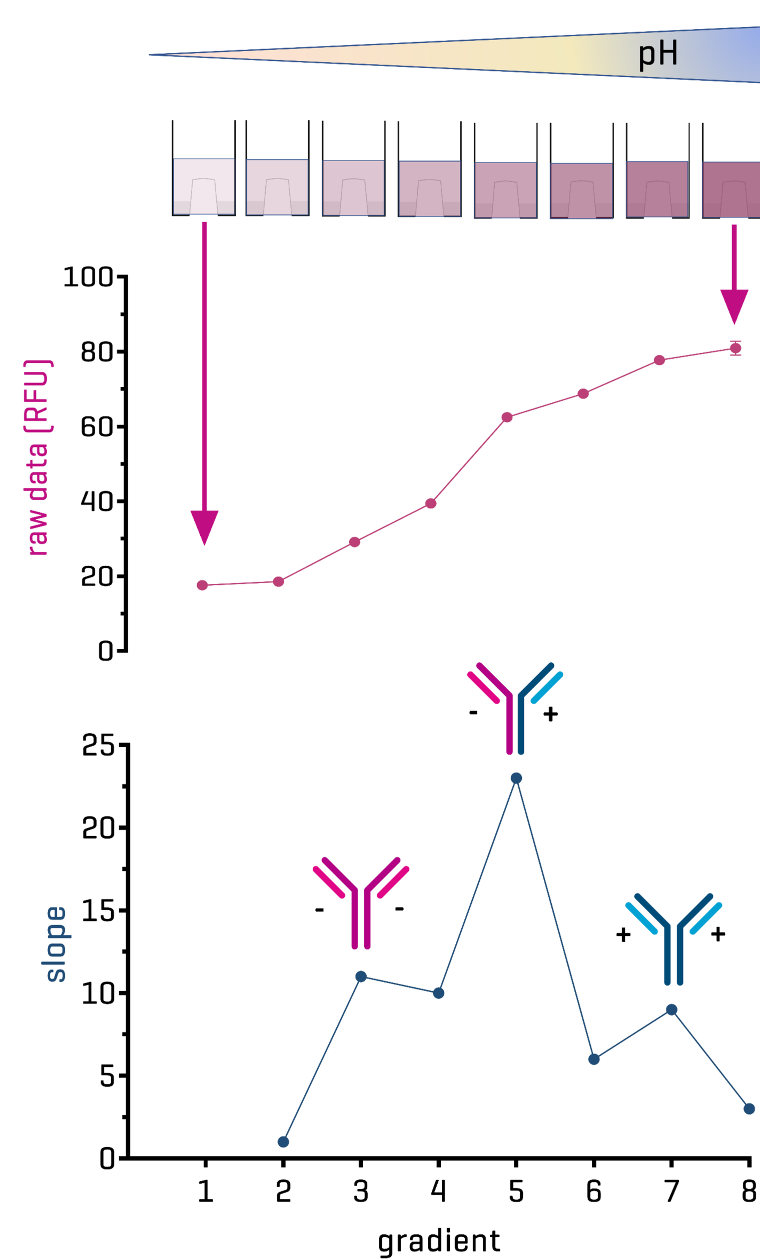

Figure 2. Assay data analysis for the PA-401 assay
The release of the analytes is measured in a fine pH gradient comprising eight neighboring wells with increasing pH (**top**), producing a raw data curve with increasing fluorescence (**middle**). Molecules with differences in surface charge/isoelectric point (pI) are released at different gradient points and this produces an increase in intensity at different gradient positions. Calculation of the slope of the raw data curve generates a chromatogram-like charge profile with peaks for the different molecules in a mixture (**bottom**).

Materials and methods

The PAIA Purity and Mispairing Assay (Art.-No. PA-401) was performed according to the standard protocol. The kit contains the ready-to-use PAIA*plate* with capture beads and the pH gradient dried in. 50 µL of reagent were added to each well of the 384-well PAIA*plate* and 8 x 10 µL of the sample was distributed into eight wells with different pH. After that, the PAIA*plate* was shaken for 45 minutes on an orbital shaker at 1800 rpm. After sedimentation of the beads the plates were measured at 635/660 nm on a fluorescence plate reader in the bottom reading mode.

The total analyte concentrations in the samples were at 50 µg/mL for the measurement of reference molecules and at 10 - 50 µg/mL for the bispecific biosimilar experiments.

CEX chromatography was performed on a Column Thermo ProPac WCX-10, 250 x 4 mm column with UV detection at 280 nm. The Thermo CX-1 pH gradient buffer system was used with a gradient starting at 30% buffer B, reaching 70% buffer B after 15 minutes.

Comparison with cation exchange chromatography

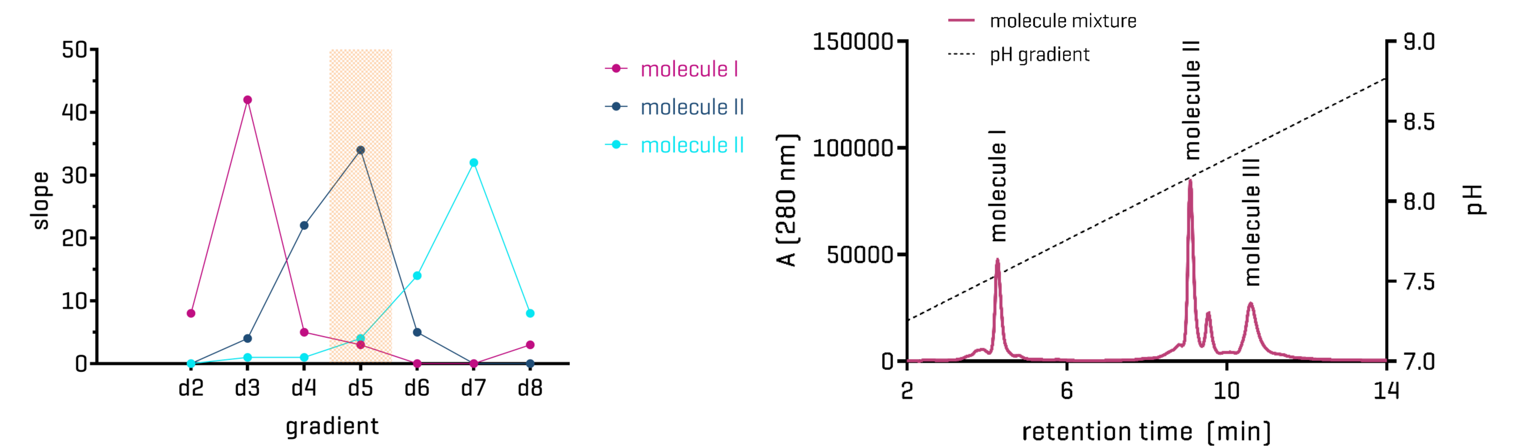

Figure 3. Comparison of reference mAb charge profiles generated with the PAIA chain mispairing assay and CEX chromatography
Three reference mAbs were analysed with both methods. The peak positions of the three mAbs in the PAIA assay (**left**) corresponds well with the retention times on the CEX chromatogram (**right**). Molecule I elutes first and molecule III last.

Analysis of mixtures of reference antibodies

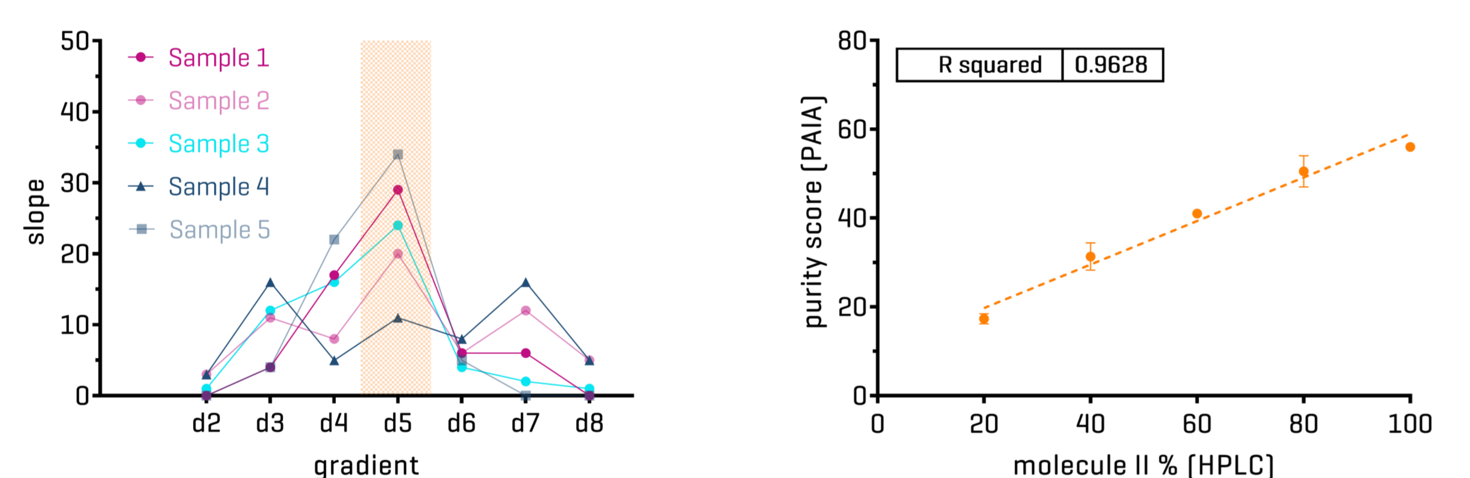

Figure 4. Quantitative analysis of ternary mixtures of reference mAbs
Mixtures of the references were generated and measured at a concentration of 50 µg/mL (**left**). The resulting profiles show distinct peaks for the reference molecules. We then calculated the *purity score*, a relative number for purity, from the slope at gradient point 5 and plotted it against the expected "true" content of molecule II in the mixtures (**right**).

Screening of stable pool samples for bispecific content

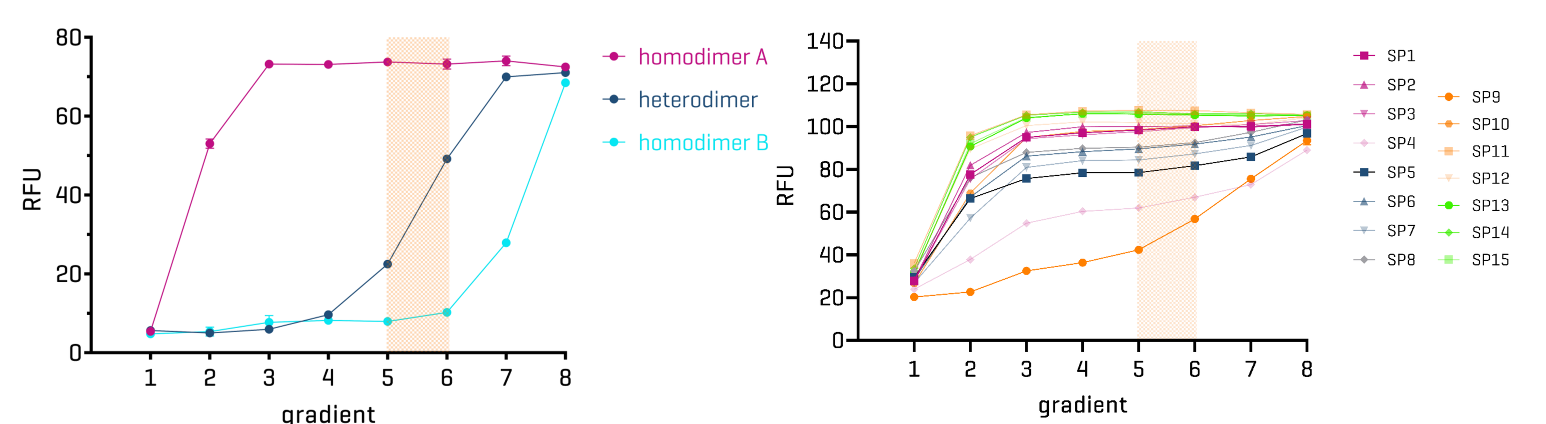

Figure 5. Charge profiles of the purified bispecific (heterodimer), the homodimers and the stable pool samples.
Charge profiles of the bispecific and the two main byproducts, the homodimers A and B (**left**). Screening of 15 diluted samples from stable pools (**right**). SP9 contains the highest amount of bispecific as evidenced by the signal increase at gradient points 5-6, followed by sample SP4. Evaluation of the slopes resulted in bispecific purity of 54% for SP9 and 24% for SP4. The profiles also reveal that the homodimer A is the main byproduct.

Conclusions

- We have transferred the principle of CEX into a microplate and developed the first commercial assay kit for the quantitative analysis of mixtures of differently charged proteins.
- The assay can measure the purity of bispecifics and other complex biologics in diluted cell culture samples. Sample purification is therefore not necessary.
- The titer of the diluted samples can be as low as 2~5 µg/mL.
- 48 samples can be analyzed with one PA-401 purity and mispairing assay kit in less than 1 hour.