DIA-Umpire[2] for protein identifications on these samples), which suggests that the time-consuming generation of tailored libraries or the addition of external retention-time (RT) calibration standards may become superfluous as more spectral libraries become publicly available.

Although here we contrast the sensitivity of targeted extraction tools with MSPLIT-DIA, these are in fact complementary approaches (**Fig. 1f** and **Supplementary Fig. 8**). Targeted extraction tools performed relatively well on libraries generated from the paired DDA samples (with matched complexity, instrument parameters and chromatographic resolution; **Supplementary Fig. 2**), but this was not the case with the large, generic SWATHAtlas library[5] (**Fig. 1g** and **Supplementary Fig. 9**). First, we showed that MSPLIT-DIA greatly facilitated targeted extraction by assisting in RT alignment without the need for spike-in standards, as peptides identified by MSPLIT-DIA in the DIA run served as markers for alignment (**Fig. 1g** and **Supplementary Fig. 9**). Second, restricting the targeted quantification search space to only MSPLIT-DIA–identified peptides yielded much smaller assay libraries that either enabled (Skyline) or systematically improved (PeakView[6], OpenSWATH[1]) targeted extraction results (**Supplementary Fig. 9**). All together, these processes substantially simplified the targeted extraction of quantitative data for up to 88% of the peptides identified by MSPLIT-DIA without affecting the reproducibility of the quantification of these newly identified peptides (**Supplementary Fig. 10**). Assay libraries for targeted extraction tools are automatically generated by MSPLIT-DIA to facilitate coupling of sensitive identification with accurate quantification from DIA data.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper (doi:10.1038/nmeth.3655).

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AUTHOR CONTRIBUTION

J.W. and N.B. developed MSPLIT-DIA; J.W. implemented the software; B.L. and M.T. acquired mass spectrometry data; J.D.R.K., B.L., S.T. and J.-P.L. helped with data analysis; N.B. and A.-C.G. supervised the project; J.W., A.-C.G. and N.B. wrote the manuscript with input from all other authors.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper (doi:10.1038/nmeth.3655).

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ANOVA and the analysis of drug combination experiments

To the Editor: In a recent *Nature Methods* Points of Significance piece[1], drug combination was used to illustrate the principles of factorial experiments for the analysis of interaction effects. Factorial analysis of variance (ANOVA) can be very misleading in drug combination studies. Drugs follow a nonlinear dose-response pattern, and ANOVA is based on linear modeling. In practical terms, this means that unless the doses chosen in an experiment are in the linear-response range for the drugs, ANOVA might not detect a drug interaction. For example, if one dose for one of the drugs is at saturation response, then the data might seem to show a negative interaction (inhibition) for a drug that in reality has additive effects.

Nonlinearity is a general problem for factorial ANOVA for several types of variables. This can be dealt with in many cases by the use of pilot studies to establish the linear-response range. However, this is often not possible in drug studies, where random effects can cause minor but significant shifts in response curves between experiments, such that the linearity assumption cannot be made. To overcome this, it is best to study drug interactions in experiments that generate response curves for the drugs both individually and in combination in the same experimental replicate. Data from these types of experiments can be used in a variety of appropriate analyses such as isobologram and combination index[2], curve shift[3] and nonlinear mixed effect[4] analyses. An additional advantage of these methods is that they allow for quantification of the strength of the interaction between drugs, which is crucial for practical decision making in drug combination experimental design.

It is important for researchers to be aware of the pitfalls of factorial experimental designs in the study of drug combination. There is a large and growing literature on the interpretation of degrees of drug synergy (positive interaction) using these methods[2–5]. Recent advances include the application of nonparametric methods as well as more precise consideration of the specific nonlinear forms of response curves and the relative potency of the two drugs being investigated[6]. Now that computationally intense methods are available to all with access to a personal computer, there is no reason not to use more robust and informative methods.

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