5 years later: irisin detection still an issue

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Since the discovery of irisin in early 2012 (1), considerable attention has been directed toward understanding the physiological role of this circulating myokine in humans. Animal studies demonstrating important metabolic benefits have been convincing (1, 2), but whether these findings translate to humans remains unclear, and many discrepant results have been reported (3, 4, 5). A tremendous limiting factor continues to be the quality and accuracy of existing commercial enzyme-linked immunosorbent assays (ELISAs) for quantifying circulating irisin levels. With growing interest in this field, resulting in over 500 publications since 2012, we feel it is important to reinforce some of the limitations of the currently available assays and the necessary steps forward, as well as share our own concerning results regarding the Phoenix Pharmaceuticals and Adipogen ELISAs, two of the most widely used assays to date (3).

Our group intended to quantify circulating irisin levels in young healthy adults in response to exercise; however, our own validation testing with both ELISAs has been less than optimal. Using the 1st-generation Phoenix Pharmaceuticals EK-067-29 assay, we observed very high coefficients of variation (CVs) of 13.2% and 13.8% for 2 separate human plasma samples (17 duplicates each, loaded interchangeably across the plate). Phoenix Pharmaceuticals subsequently improved the specificity of the biotinylated peptide in the 2nd generation of this assay (current version), yielding concentrations much closer to the 'true' circulating irisin levels, determined using tandem mass spectrometry (6). While this seemed a positive development, we remain dissatisfied with the performance of this assay, reporting CVs of 15.2% and 11.0% for 2 samples (13 duplicates each). We further tested the same 2 samples with the Adipogen ELISA (AG-45A-0046YEK-KI01) (10 duplicates each) and observed even higher CVs of 21.4% and 11.1% (Table 1). It should be emphasized that the irisin concentrations obtained

from the Adipogen assay are considerably higher than the expected range (6): irisin values measured with the Adipogen assay were 477-fold higher than those of the Phoenix assay in the same individual (Table 1).

Irisin secretion is thought to be regulated by exercise; however, many studies have reported contradictory results (3). This is not surprising given the high levels of inter- and intra-assay variation with these assays. In our lab, we observed large differences in the percent increase in irisin levels after acute maximal exercise in 4 healthy individuals between the original EK-067-52 assays ($30.8 \pm 27.6\%$ increase) (7) and 2 different EK-067-29 assays within the same lot ($2.6 \pm 10.2\%$ and $16.2 \pm 34.5\%$) (single freeze-thaw cycle). Montes-Nieto *et al.* have also reported issues with the EK-067-29 assays, showing inconsistent results between the 1st- and 2nd-generation versions: a relationship between excess weight and irisin levels was noted with the 1st-, but not the 2nd-generation assay (8). We also observed notable differences in the relative increase detected with the Phoenix Pharmaceutical and Adipogen ELISAs (7 vs 18%, respectively) in the same sample (Table 1). Other groups have relayed similar concerns regarding the performance of the existing ELISAs (3, 5, 9); yet, most groups continue using these assays without appropriate validation.

Results from a recombinant spike and recovery test conducted in-house by Phoenix Pharmaceuticals using the EK-067-29 assay (personal communication) provide further corroborating evidence for these discrepant findings and other inconsistencies in the literature to date. Percent recovery at 2 concentrations of recombinant irisin was 70% (1 ng/mL) and 115% (2 ng/mL), falling outside the commonly accepted range of 90–110% (10). The source of this variability remains unclear. Unfortunately, validation of the specificity of the antibody by mass spectrometry analysis using human plasma/serum samples is currently lacking, as only spiked recombinant irisin has been

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Table 1 Intra-assay validation testing using Pheonix Pharmaceuticals (2nd generation) and Adipogen ELISAs.

Duplicate	Phoenix pharmaceuticals EK-067-29 (2nd generation)		Adipogen AG-45A-0046YEK-KI01	
	Sample A (pre-exercise) irisin concentration (ng/mL)	Sample B (post-exercise) irisin concentration (ng/mL)	Sample A (pre-exercise) irisin concentration (ng/mL)	Sample B (post-exercise) irisin concentration (ng/mL)
1	11.17	12.50	4724.10	6846.10
2	12.99	12.56	4578.00	6276.50
3	10.80	11.25	5091.80	6719.30
4	13.33	13.17	5874.80	6108.20
5	13.44	13.67	4964.40	6164.00
6	12.83	14.80	8455.60	6403.60
7	8.97	9.58	5608.70	5822.20
8	12.86	14.17	4991.20	7264.60
9	12.10	13.38	5516.20	8412.30
10	13.56	13.84	7123.50	6910.90
11	9.17	12.52	-	-
12	14.25	13.41	-	-
13	9.47	11.33	-	-
Range	(8.98–14.25)	(9.58–14.80)	(4578.0–8455.6)	(5822.2–8412.3)
Mean (s.d.)	11.92 (1.81)	12.78 (1.41)	5692.90 (1216.38)	6692.77 (744.74)
CV	15.2%	11.0%	21.4%	11.1%
Mean relative increase post-exercise	8 ± 11%		22 ± 25%	

assessed to date (2, 4). Therefore, whether this antibody is truly specific for the different fragments of endogenous irisin has not been established.

While the Phoenix Pharmaceutical and Adipogen ELISAs are most commonly used (3), many other commercial assays utilizing polyclonal antibodies are available (Table 2). Differences in the reported range of irisin levels (0.02–4300 ng/mL) (11, 12) and level of validation testing limit comparison between studies. These issues compromise our ability to extract accurate information about irisin regulation in humans as well as its role in metabolic health and other disease states. Through personal communication with various commercial assay vendors and manufacturers, we obtained additional information regarding the validation testing performed for each of the assays (Table 2). Although most commercial assays have undergone a series of in-house validation tests and have released results that meet acceptable standards, we have demonstrated significant differences in our validation tests with the Phoenix Pharmaceutical and Adipogen assays. This unfortunately is not uncommon: variable standards in antibody validation by both suppliers and researchers have triggered a widespread ‘reproducibility crisis’ (13), leading to conflicting results and a waste of time and money. Therefore, we would urge researchers to be cautious and conduct validation testing before running samples for study analyses.

Developing a monoclonal antibody specific for irisin is an important research priority. Both Phoenix Pharmaceuticals and Adipogen assays are competitive ELISAs with polyclonal antibodies, which are known to be less sensitive and specific, compared to a sandwich ELISA (14). In late 2016, Aviscera Bioscience developed the first sandwich ELISA with a monoclonal antibody; however, they have not verified the specificity of the antibody against human irisin in plasma/serum samples with Western blot or other established methods (personal communication). Moreover, neither the recovery nor the sensitivity of the Aviscera Bioscience assay has been evaluated, and the intra- and inter-assay reproducibility has not been appropriately assessed with human plasma/serum samples.

Overall, numerous inconsistencies in the literature underscore the need to develop more accurate methods and perform more rigorous validation testing with different assays, ensuring the results meet established standards. We urge other researchers to consider these arguments; otherwise, we risk introducing further controversy and uncertainty by using unreliable assays. Confirming whether irisin will live up to its expectations in *Men’s Fitness* as a ‘Miracle Weight Loss Pill (that) Allows for Easy Workouts’ (15) will require more reliable methodologies and scientific rigor than currently exists.

Table 2 Validation testing results from commercial irisin ELISAs.

Company	Location	Catalog number	Antibody type	Intra-assay variation	Inter-assay variation	Sensitivity	Detectable range	Specificity	Spike and recovery testing	Linearity of dilutions	Western blot
Phoenix Pharmaceuticals	Burlingame CA, USA	EK-057-29 (2nd generation)	Polyclonal (Competitive ELISA)	<10% (4x/sample)	<8% (3 batches)	1.29 ng/mL	1.29–27.5 ng/mL	Minimal cross-reactivity with FND5 (9%)	70–115%	Not tested	Antibody was tested in-house with recombinant protein, but not endogenous irisin from plasma or serum samples
Adipogen Distributor: Biovendor	San Diego, CA, USA Czech Republic	AG-45A-0046YEK-K101 RAG018R	Polyclonal (Competitive ELISA)	<9% 6 human samples (8 replicates)	<10% 5 human samples tested across 5 assays	1 ng/mL	1–5000 ng/mL	No cross-reactivity with irisin analogues	Human serum samples (n=3) Range: 85–125%	Human serum samples (n=4) Range: 90–107%	Tested in human plasma samples by Jedrychowski et al. – 12 kDa band was detected (6)
Cusabio	Wuhan, China	CS8-EQ027943HU	Polyclonal (Competitive ELISA)	<8% 3 samples (20 replicates each)	<10% 3 samples (20 replicates each)	0.78 ng/mL	3.12–200 ng/mL	'We tested the cross-reactivity with rat and mouse and there is no cross-reactivity' (personal communications)	Serum (n=5): 94–106% EDTA plasma (n=4): 101–112%	Tested 1:2, 1:4, and 1:8 Range: 83–109%	Not tested with serum or plasma samples
Hangzhou Eastbiopharm	Hangzhou, China	SL2020Hu	Polyclonal (Competitive ELISA)	≤4.3%	≤8.5%	0.12 ng/mL	78.0 pg/mL–5000 pg/mL	'This assay recognizes recombinant and natural human FND5. No significant cross-reactivity or interference was observed'	Serum: 95–102% Plasma: 96–103%	Tested 1:2, 1:4, 1:8, and 1:16 EDTA plasma (n=5): 91–103% Serum (n=5): 92–103% Heparin plasma (n=5): 89–105%	A673 cell lysate was tested with FND5 antibody: observed molecular weight=24 kD. Not tested using endogenous irisin from human plasma/serum
Cloud-Clone Corp USCN Life Science	Wuhan, China	SEN576Hu	Polyclonal (Competitive ELISA)	<10% only standards were tested (low, medium, high)	<12% only standards were tested (low, medium, high)	6.9 pg/mL	15.6–1000 pg/mL	'No significant cross-reactivity or interference between FND5 and analogues was observed, however possible cross-reactivity may still exist'	Plasma: 81–95% Serum: 80–92%	Tested samples spiked with FND5 at 1:2, 1:4, 1:8, and 1:16 (n=5) Range: 85–105%	Antibody was tested using WB in tissue, but not endogenous irisin from plasma or serum samples
Aviscera Bioscience	Santa Clara, CA, USA	SK00170-09	Monoclonal (Sandwich ELISA)	Not tested in plasma/serum samples, only using standards (4–6%)	Not tested in plasma/serum samples, only using standards (8–10%)	0.1 ng/mL	0.4–51.2 ng/mL	Not tested	Tested 1:8 and 1:16	Not tested	Antibody was tested with recombinant protein, but not endogenous irisin from plasma or serum samples

ELISA, enzyme-linked immunosorbent assay; FND5, fibronectin type III domain-containing protein 5; WB, Western blot.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the commentary reported.

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