Program # LB180

Reproducibility of Phosphoinositide Lipid Extraction & Presentation in Bioanalytical Assays

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1. Abstract

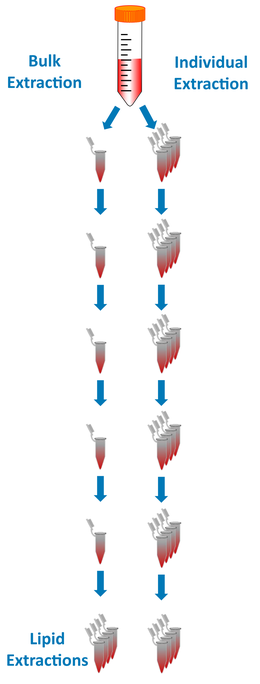
Phosphatidylinositol 4-phosphate (PI(4)P) produced by Phosphatidylinositol 4-Phosphate kinases is an abundant Golgi phosphoinositide functioning in lipid exchange and vesicle transport within mammalian cells. Researchers wanting to quantify PI(4)P use the Bligh and Dyer method for extracting and separating phospholipids in cells and biological tissues. This common lipid extraction procedure is known to have inherent variation. Using a human promyelocytic leukemia cell line and an ELISA that is sensitive and specific for PI(4)P, we examine sources of variation in bioassays including sample (HL-60 cells), the extraction procedure, and lipid detection. We determined a PI(4)P Mass ELISA has low coefficient of variation (<10%) if the extracted sample volume is held constant and samples are prepped and lipids presented with standardized procedures. Thus, the organic solvent extraction process is the major source of variation in typical Bligh-Dyer preparations.

2. Overview

- Phosphatidylinositol 4-phosphate (PI(4)P) produced by PI4P kinases is an abundant Golgi phosphoinositide functioning in lipid exchange and vesicle transport.
- Common Bligh and Dyer lipid extraction is known to have inherent variation; and was evaluated in a PI(4)P quantification system.
- Used HL-60 cells and a PI(4)P ELISA to examine sources of experimental variation.

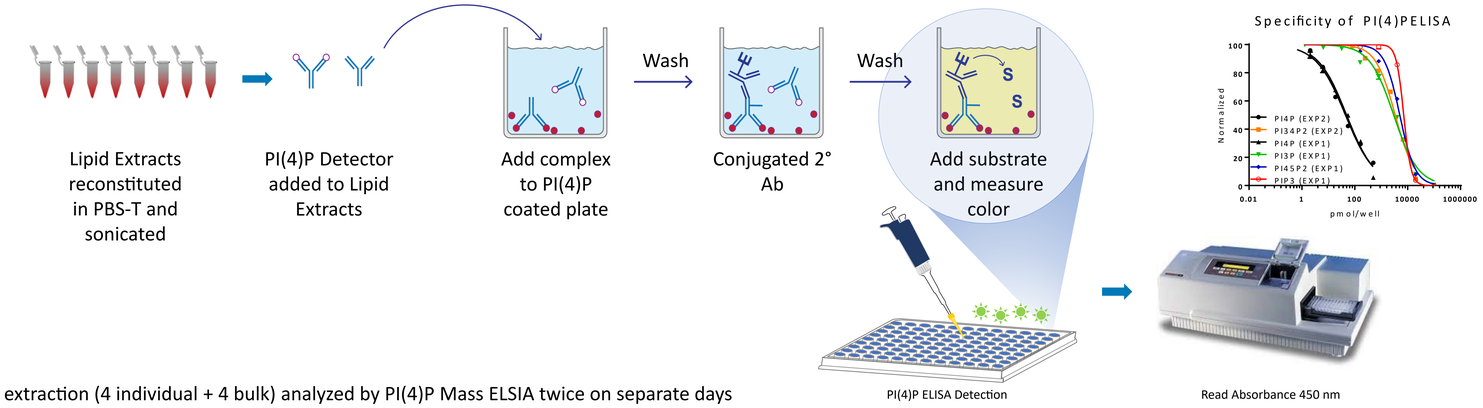
3. Extraction procedure

- Performed extraction procedure 4 times on 4 separate days
- Each extraction day produced 4 vials of "individual extraction" and 4 vials of "bulk extraction"
- Procedures performed in polypropylene tubes at room temperature



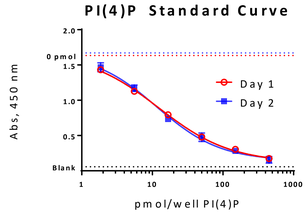
1. Collect HL-60 (promyelocytic leukemia) cells and centrifuge. Remove media. Add cold 0.5 M TCA to a concentration of 2 X 10^6 cells/mL.
2. Aliquot into 5mL tubes. Centrifuge. Discard supernatant.
3. Wash pellet with TCA/ 1 mM EDTA. Vortex. Centrifuge. Discard supernatant. Repeat one time.
4. Extract neutral lipids with MeOH:$CHCl_3$ (2:1) and vortex for 10 minutes. Centrifuge. Discard supernatant. Repeat one time.
5. Extract acidic lipids with MEOH:$CHCl_3$:12 N HCl (80:40:1) and vortex for 25 minutes. Centrifuge. Transfer supernatant to a new vial. Discard pellet.
6. Phase split. To supernatant from step 4, add $CHCl_3$ followed by 0.1 N HCl. Vortex 30 seconds. Centrifuge to separate organic and aqueous phases.
7. Collect the organic (lower) phase, dry and store at -20 °C until PI(4)P ELISA testing.

4. PI(4)P Detection



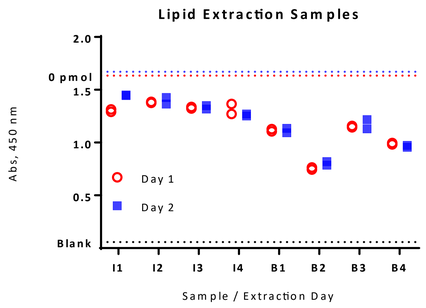
- Each extraction (4 individual + 4 bulk) analyzed by PI(4)P Mass ELSIA twice on separate days
- Higher concentrations of PI(4)P in a sample-extract bind to PI(4)P detector protein in solution and prohibit protein binding to PI(4)P immobilized on plate surface
- Results from both individual and bulk collected samples were compared for intra- and inter- assay sample coefficient of variation (CV)
- ELISA is selective for PI(4)P over other phosphoinositides and lipids that may also be present in extracted samples

5. Intra Assay CV



Sample:
I = Individual
B = Bulk

Extraction Day:
1-4

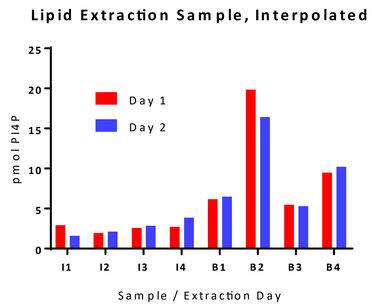


- Intra assay variation of 9.2% with a range of 2.6%-32.4%.

Day 1					
Lipid Extracton	pmol/well		Average	CV	Avg CV
I1	3.10	2.76	2.93	8.3%	
I2	2.00	1.90	1.95	3.7%	
I3	2.66	2.47	2.57	5.3%	
I4	3.37	2.12	2.75	32.4%	12.4%
B1	5.94	6.40	6.17	5.3%	
B2	20.48	19.22	19.85	4.5%	
B3	5.59	5.33	5.46	3.4%	
B4	9.66	9.29	9.47	2.7%	4.0%

Day 2					
Lipid Extracton	pmol/well		Average	CV	Avg CV
I1	1.57	1.63	1.60	2.6%	
I2	1.80	2.49	2.15	22.8%	
I3	2.66	3.09	2.87	10.6%	
I4	3.74	3.99	3.87	4.6%	10.2%
B1	6.91	6.06	6.48	9.3%	
B2	17.25	15.62	16.44	7.0%	
B3	6.12	4.56	5.34	20.6%	
B4	9.96	10.48	10.22	3.5%	10.1%

6. Inter Assay CV



- Inter assay variation of **6%** with a range of 2.1%-13.3% (bulk lipid extractions).
- Combined sample and assay inter variation of **20%,** with a range of 6.3% - 41.4% (individual extraction).

Interpolated Data					
	pmol PI(4)P/well				
Lipid Extracton	3/20/2018	3/22/2018	Average	CV	Avg CV
I1	2.92	1.60	2.26	41.4%	
I2	1.95	2.13	2.04	6.3%	
I3	2.57	2.87	2.72	7.9%	
I4	2.71	3.86	3.29	24.9%	20.1%
B1	6.17	6.47	6.32	3.4%	
B2	19.84	16.42	18.13	13.3%	
B3	5.46	5.30	5.38	2.1%	
B4	9.47	10.22	9.84	5.4%	6.0%

7. Conclusions

- Determined the PI(4)P Mass lipid detection portion of the procedure has a low coefficient of variation (<10%).
- Lipid extractions with organic solvent is the major source of variation in typical Bligh-Dyer preparations with CV about 20%.
- Careful technique and practice with small-volume liquid/liquid organic extractions will improve accuracy when quantifying phosphoinositides from biological samples.

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