

Role of Housekeeping Proteins in PBMC Fit-for-Purpose Validation: PARK7 Reveals Lysis-Dependent Bias in BRD4 Quantification by Mass Spectrometry

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PURPOSE

Traditional protein-based analysis methods, such as Western blotting, often struggle to distinguish true biological changes from sample loading variability. In contrast, mass-spectrometry-based proteomics provides multiplexed measurements, enabling the relative and absolute quantification of thousands of proteins across complex biological systems. This broad coverage not only enhances biological interpretation but also allows the identification of stably expressed proteins directly from the dataset¹.

When combined with a direct digestion approach, MS thus offers an advantage over antibody-based assays like LBA by revealing endogenous proteins that can serve as more reliable loading controls than conventional strategies such as BCA assays, especially in samples prone to red blood cell contamination.

PARK7² is a highly conserved multifunctional enzyme found across species, from prokaryotes to eukaryotes. Its enzymatic and non-enzymatic functions (e.g., anti-oxidation, anti-glycation, protein quality control), along with its role as a transcriptional coactivator, support its involvement in key cellular processes such as epigenetic regulation, making it a promising therapeutic target for diseases including cancer and Parkinson's disease³.

Bromodomain-containing protein 4 (BRD4), a key transcriptional coactivator, drives cancer and inflammation through both bromodomain-dependent and independent mechanisms. Resistance to bromodomain inhibitors has prompted alternative strategies, including BRD4-targeted degraders, non-bromodomain ligands that disrupt scaffolding functions, PTM disruptors, and condensate modulators⁴.

Here, we present a case study of fit-for-purpose validation (FFPV) for BRD4 quantification in PBMCs using PARK7 as a normalizing protein. Key steps—including PBMC collection, lysis, homogenization, and digestion—were assessed as potential sources of variability. This work highlights the need to tailor sample preparation to both the analyte and normalizer to ensure robust performance.

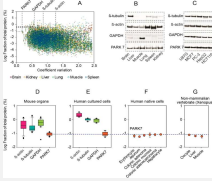


Figure 1. PARK7 is a ubiquitous protein with constant expression in vertebrate cells. ¹

METHODS

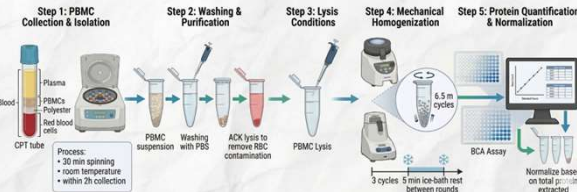


Figure 2. Sample Preparation from PBMC Collection to BCA total protein quantization.

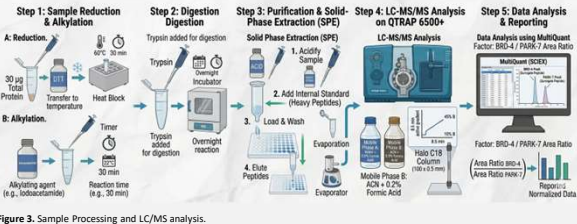


Figure 3. Sample Processing and LC/MS analysis.

RESULTS

1. Parallelism – Original Assay

Parallelism assessment of PBMC isolations performed from varying cell inputs revealed inconsistent outcomes. Normalized BRD4 levels did not display a consistent trend, with responses either improving or deteriorating depending on the donor. These findings prompted a thorough re-evaluation of the entire workflow, from PBMC collection and stability through homogenization, total protein processing, and digestion efficiency. Pinpointing the specific steps at which the two target proteins diverge in behavior is critical to control the assay performances and successfully validate the method.

Table 3. Parallelism for the number of processed cells – Original assay

PARK7	Donor 1			Donor 2			Donor 3		
	2X Cells	1X Cells	1X Cells	2X Cells	1X Cells	1X Cells	2X Cells	1X Cells	1X Cells
Area Ratio	18.1474	21.2114	24.1146	26.8097	20.4082	24.1146	18.1474	21.2114	24.1146
Mean	18.1474	21.2114	24.1146	26.8097	20.4082	24.1146	18.1474	21.2114	24.1146
%CV	3.1	1.9	6.1	4.8	2.2	4.5	3.1	1.9	6.1
%Difference	-	-	-	-	-	-	-	-	-

Table 4. Parallelism for the amount of material loaded during sample processing

BRD4	Donor 1			Donor 2			Donor 3		
	2X Cells	1X Cells	1X Cells	2X Cells	1X Cells	1X Cells	2X Cells	1X Cells	1X Cells
Area Ratio	0.0546	0.0683	0.0481	0.0455	0.0590	0.0648	0.0546	0.0683	0.0481
Mean	0.0546	0.0683	0.0481	0.0455	0.0590	0.0648	0.0546	0.0683	0.0481
%CV	9.4	8.6	4.8	15.1	4.3	9.3	9.4	8.6	4.8
%Difference	-	-	-	-	-	-	-	-	-

Table 5. Parallelism for the amount of material loaded during sample processing

BRD4 Normalized	Donor 1			Donor 2			Donor 3		
	2X Cells	1X Cells	1X Cells	2X Cells	1X Cells	1X Cells	2X Cells	1X Cells	1X Cells
BRD4 Area Ratio vs. PARK7	0.0029	0.0032	0.0020	0.0017	0.0029	0.0027	0.0029	0.0032	0.0020
Area Ratio	0.0033	0.0029	0.0022	0.0014	0.0029	0.0028	0.0033	0.0029	0.0022
Mean	0.0033	0.0029	0.0022	0.0014	0.0029	0.0028	0.0033	0.0029	0.0022
%CV	12.1	11.0	4.8	13.9	2.0	11.1	12.1	11.0	4.8
%Difference	-	-	-	-	-	-	-	-	-

3. Parallelism – Updated Assay

Consistent results across different cell numbers and processing amounts were achieved using the surfactant-based assay.

Table 4. A) Parallelism for the number of processed cells during PBMC isolation; B) Parallelism for the amount of material loaded during sample processing

A) PARK7	5X Cells			2.5X Cells			1X Cells		
	Area Ratio	Mean	%CV	Area Ratio	Mean	%CV	Area Ratio	Mean	%CV
Area Ratio	0.5020	0.4920	0.4588	0.5020	0.4920	0.4588	0.5020	0.4920	0.4588
Mean	0.4941	0.5031	0.4907	0.4941	0.5031	0.4907	0.4941	0.5031	0.4907
%CV	0.5097	0.5021	0.4865	0.5097	0.5021	0.4865	0.5097	0.5021	0.4865
%Difference	-	-	-	-	-	-	-	-	-

Table 5. Parallelism for the number of processed cells during PBMC isolation; B) Parallelism for the amount of material loaded during sample processing

B) PARK7	25ug			12.5ug			6.25ug		
	Area Ratio	Mean	%CV	Area Ratio	Mean	%CV	Area Ratio	Mean	%CV
Area Ratio	2.4102	0.5020	2.2619	2.4102	0.5020	2.2619	2.4102	0.5020	2.2619
Mean	2.3348	0.4941	2.1867	2.3348	0.4941	2.1867	2.3348	0.4941	2.1867
%CV	2.3810	0.5097	2.2713	2.3810	0.5097	2.2713	2.3810	0.5097	2.2713
%Difference	-	-	-	-	-	-	-	-	-

Table 6. Parallelism for the number of processed cells during PBMC isolation; B) Parallelism for the amount of material loaded during sample processing

BRD4	5X Cells			2.5X Cells			1X Cells		
	Area Ratio	Mean	%CV	Area Ratio	Mean	%CV	Area Ratio	Mean	%CV
Area Ratio	0.0564	0.0470	0.0600	0.0564	0.0470	0.0600	0.0564	0.0470	0.0600
Mean	0.0572	0.0496	0.0507	0.0572	0.0496	0.0507	0.0572	0.0496	0.0507
%CV	0.0587	0.0409	0.0515	0.0587	0.0409	0.0515	0.0587	0.0409	0.0515
%Difference	-	-	-	-	-	-	-	-	-

Table 7. Parallelism for the number of processed cells during PBMC isolation; B) Parallelism for the amount of material loaded during sample processing

Norm. BRD4	5X Cells			2.5X Cells			1X Cells		
	Area Ratio	Mean	%CV	Area Ratio	Mean	%CV	Area Ratio	Mean	%CV
Area Ratio	0.1058	0.0919	0.1176	0.1058	0.0919	0.1176	0.1058	0.0919	0.1176
Mean	0.1128	0.1092	0.1105	0.1128	0.1092	0.1105	0.1128	0.1092	0.1105
%CV	0.1128	0.0953	0.1110	0.1128	0.0953	0.1110	0.1128	0.0953	0.1110
%Difference	-	-	-	-	-	-	-	-	-

2. Assay Troubleshooting

Experimental data indicate that the only point where the 2 protein differs is the PBMC lysis. The denaturant-based homogenization protocol yields the highest total protein recovery but the lowest recovery for BRD4. In contrast, surfactant-based homogenization approaches improve analyte recovery and enable more consistent normalization of BRD4 relative to PARK7. Based on overall performance, Rapigest was selected for further validation, as it provided improved sensitivity for BRD4 compared to the other conditions.

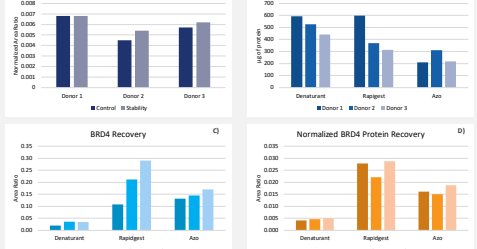


Figure 6. A) Stability test of normalized BRD4 in PBMC with 2h benchtop stability at 5°C post centrifugation; B) Total protein recovery per homogenization condition; C) BRD4 recovery per tested homogenization condition; D) Normalized BRD4 recovery per homogenization condition

4. Representative Chromatograms – Updated Assay

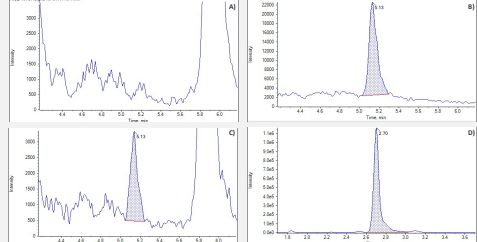


Figure 4. A) Blank for BRD4; B) Endogenous concentration of BRD4 in lysed PBMC; C) 6.34 pg/ug for BRD4 (LOQ); D) Endogenous concentration of PARK7.

5. Validation Results – Selectivity

Due to their endogenous presence, the selectivity of the two biomarkers is assessed using the peak area ratio of the quantifier to qualifier MRM transitions in buffer vs. extracted matrix samples. The percent difference between the transition ratios in matrix and buffer must not exceed 30.0%.

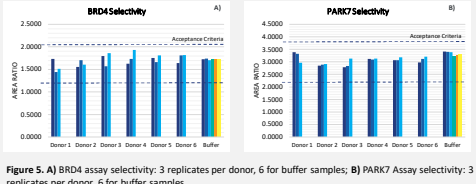


Figure 5. A) BRD4 assay selectivity: 3 replicates per donor, 6 for buffer samples; B) PARK7 assay selectivity: 3 replicates per donor, 6 for buffer samples.

6. Validation Results – Assay Range

Linearity testing of the SIL peptide response indicated a linear response across the 125.0 to 12500.0 fmol/mL range, meeting the sensitivity requirements for this assay.

Table 1. Linearity Testing for PARK7

PARK7	Reversed Curve STD Calculated Concentrations (fmol/mL)									
	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	STD 9	STD 10
Normal Concentration	31.3	62.5	125.0	250.0	375.0	750.0	1125.0	12500.0	12500.0	12500.0
Calculated Concentration	RC (20.1)	RC (71.1)	121.7	273.0	2019.4	3686.2	9093.4	11306.5	12206.1	12206.1
Mean	RC (68.8)	RC (74.0)	120.4	260.0	1652.9	3994.4	7690.6	11037.3	11052.1	11052.1
%CV	-	-	121.09	266.50	1396.15	3940.30	8392.00	11171.90	12629.10	12629.10
%CV	-	-	0.92	9.19	259.15	217.93	991.93	150.35	598.21	598.21
%CV	-	-	0.8	3.4	14.1	5.7	11.8	1.7	4.7	4.7
%CV	-	-	9.3	6.6	2.1	5.4	-4.1	-0.7	1.0	1.0

Table 2. Linearity Testing for BRD4

BRD4	Reversed Curve STD Calculated Concentrations (fmol/mL)									
	STD-1	STD-2	STD-3	STD-4	STD-5	STD-6	STD-7	STD-8	STD-9	STD-10
Normal Concentration	31.3	62.5	125.0	250.0	375.0	750.0	1125.0	12500.0	12500.0	12500.0
Calculated Concentration	RC (33.6)	RC (66.6)	128.2	249.7	1909.3	3984.2	8749.9	11465.0	13220.1	13220.1
Mean	N/D	N/D	127.4	228.1	1889.9	3742.4	8618.0	10481.8	12150.8	12150.8
%CV	-	-	127.80	217.90	1899.40	8681.30	8681.30	10971.40	12685.65	12685.65
%CV	-	-	0.57	16.09	13.72	170.98	93.27	692.40	756.39	756.39
%CV	-	-	0.4	7.0	0.7	4.4	1.1	6.3	6.0	6.0
%CV	-	-	2.2	4.8	1.3	3.0	-0.8	-2.5	1.5	1.5

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