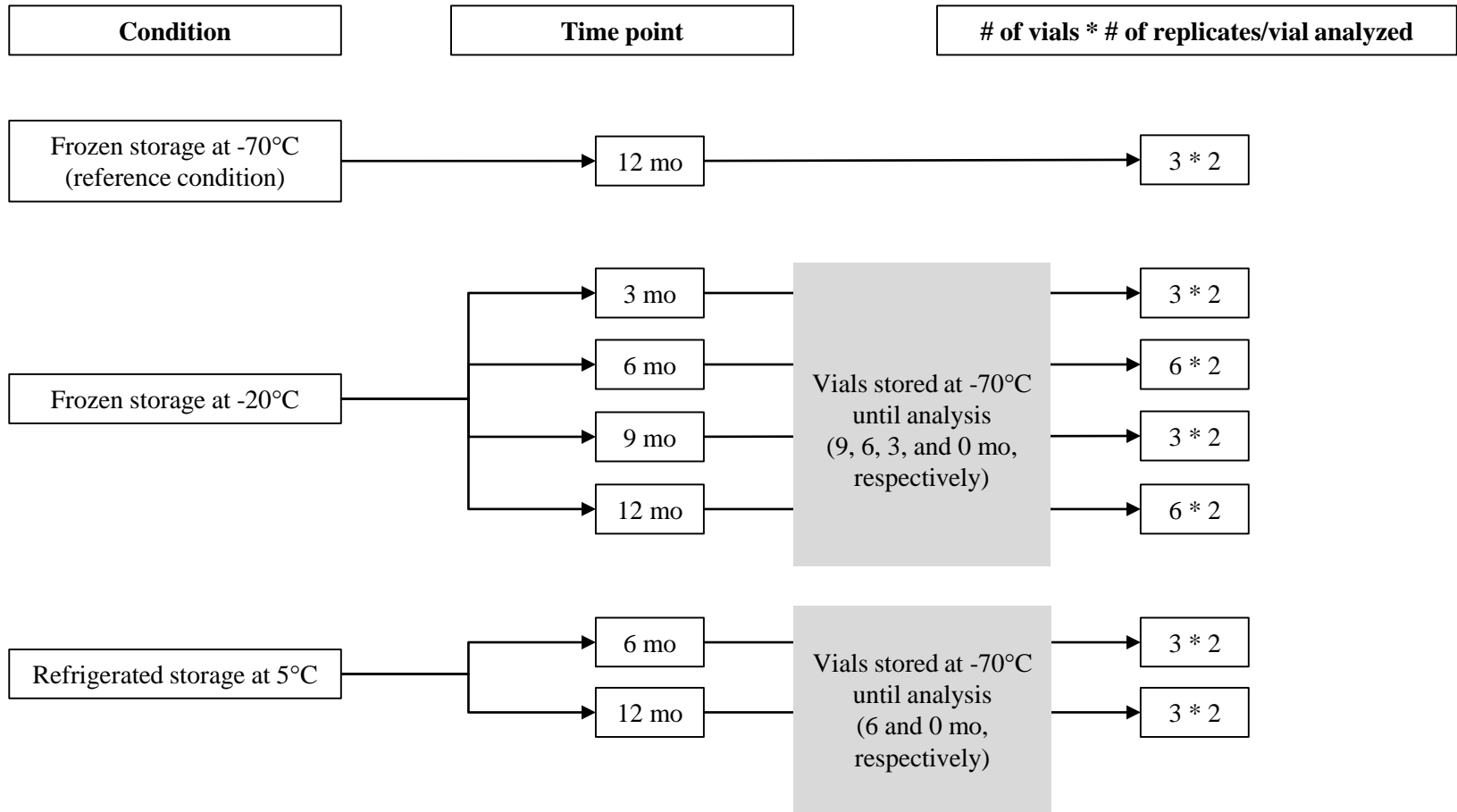


Supplemental Figure 1. Design of long-term storage study



The following serum biomarkers were analyzed: ferritin, soluble transferrin receptor, iron, unbound iron-binding capacity, C-reactive protein, folate (also in whole blood), pyridoxal-5'-phosphate, 4-pyridoxic acid, vitamin B-12, total homocysteine, methylmalonic acid, vitamin C, vitamin A, vitamin E, γ -tocopherol, 25-hydroxyvitamin D₃, and 25-hydroxyvitamin D₂.

Three pools were analyzed for each biomarker, except for C-reactive protein and vitamin C (2 pools).

No samples were available for methylmalonic acid for 3 and 9 mo storage at -20°C.

No samples were available for whole blood folate and serum vitamin C for storage at 5°C.

Supplemental Table 1. Analytical methods used and quality specifications for analytical imprecision and bias based on biologic variation^a

Biomarker	Method (<i>reference</i>)	Analytical and biologic variation ^b			Quality specifications for analytical imprecision, % ^c			Quality specifications for bias, % ^d		
		CV _A	CV _I (<i>reference</i>)	CV _G (<i>reference</i>)	Optimum	Desirable	Minimum	Optimum	Desirable	Minimum
<i>Iron-status indicators</i>										
FER	ECLIA on Roche E170® (1)	3.2%	14.2 (14)	15.0 (14)	3.6	7.1	10.7	2.6	5.2	7.7
sTfR	Immunoturbidimetric assay on Roche Mod P® (2)	3.1%	11.3 (15)	17.3 (16)	2.8	5.7	8.5	2.6	5.2	7.7
Iron	Colorimetric assay on Roche Mod P® (3)	3.3%	26.5 (14)	23.2 (14)	6.6	13.3	19.9	4.4	8.8	13.2
UIBC	Colorimetric assay on Roche Mod P® (4)	4.9%	NA ^e	NA	NA	NA	NA	NA	NA	NA
CRP	Immunoturbidimetric assay Roche Mod P® (5)	5.1%	42.2 (14)	76.3 (14)	10.6	21.1	31.7	10.9	21.8	32.7
<i>Water-soluble vitamins</i>										
S-FOL	Microbiologic assay (6)	7.7%	21.5 (17)	48.7 (17)	5.4	10.8	16.1	6.7	13.3	20.0
WB-FOL	Microbiologic assay (6)	10.3%	9.1 (17)	35.8 (17)	2.3	4.6	6.8	4.6	9.2	13.9
PLP	HPLC-Fluorescence (7)	5.2%	20.0 (18)	34.0 (18)	5.0	10.0	15.0	4.9	9.9	14.8
4-PA	HPLC-Fluorescence (7)	4.6%	NA	NA	NA	NA	NA	NA	NA	NA
B12	ECLIA on Roche E170® (8)	4.7%	14.6 (17)	43.6 (17)	3.7	7.3	11.0	5.7	11.5	17.2
tHcy	HPLC-Fluorescence (9)	4.3%	12.2 (17)	37.1 (17)	3.1	6.1	9.2	4.9	9.8	14.6
MMA	HPLC-MS/MS (10)	5.7%	18.7 (17)	41.0 (17)	4.7	9.4	14.0	5.6	11.3	16.9
VIC	HPLC-Electrochemical (11)	7.2%	26.0 (14)	31.0 (14)	6.5	13.0	19.5	5.1	10.1	15.2
<i>Fat-soluble vitamins</i>										
VIA	HPLC-UV (12)	4.5%	10.4 (17)	31.0 (17)	2.6	5.2	7.8	4.1	8.2	12.3
VIE	HPLC-UV (12)	3.9%	13.9 (17)	39.8 (17)	3.5	7.0	10.4	5.3	10.5	15.8
γ-TOC	HPLC-UV (12)	6.9%	23.2 (17)	51.0 (17)	5.8	11.6	17.4	7.0	14.0	21.0
25OHD ₃	HPLC-MS/MS (13)	4.1%	11.3 ^g (17)	37.9 ^f (17)	2.8	5.7	8.5	4.9	9.9	14.8
25OHD ₂	HPLC-MS/MS (13)	5.4%	NA	NA	NA	NA	NA	NA	NA	NA

^a 25OHD₂, 25-hydroxyvitamin D₂; 25OHD₃, 25-hydroxyvitamin D₃; 4PA, 4-pyridoxic acid; B12, vitamin B-12; CRP, C-reactive protein; ECLIA: electrochemiluminescence immunoassay; FER, ferritin; MMA, methylmalonic acid; PLP, pyridoxal-5'-phosphate; S-FOL, serum folate; sTfR, soluble transferrin receptor; tHcy, total homocysteine; γ-TOC, γ-tocopherol; UIBC, unsaturated iron-binding capacity; VIA, vitamin A (retinol); VIE, vitamin E (α-tocopherol); VIC, vitamin C (ascorbic acid); WB-FOL, whole blood folate.

^b CV_A: mean analytical imprecision from at least 20 runs and 2-3 levels of QC pools; CV_I: intra-individual variation; CV_G: inter-individual or group variation.

^c Quality specifications for analytical imprecision are based on intra-individual variation (CV_I) (19); optimum assay imprecision is $\leq 0.25 \times CV_I$; desirable assay imprecision is $\leq 0.5 \times CV_I$; minimum assay imprecision is $\leq 0.75 \times CV_I$.

^d Quality specifications for bias are based on a combination of intra-individual variation (CV_I) and inter-individual variation (CV_G) (19); optimum bias is $\leq 0.125 \times (CV_I^2 + CV_G^2)^{1/2}$; desirable bias is $\leq 0.25 \times (CV_I^2 + CV_G^2)^{1/2}$; minimum bias is $\leq 0.375 \times (CV_I^2 + CV_G^2)^{1/2}$.

^e Quality specifications for analytical imprecision and bias could not be calculated because of the lack of data for biologic variation; NA, data not available.

^f For total 25OHD measured by a radioimmunoassay.

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Supplemental Table 2. Geometric mean biomarker concentrations for samples stored at -20°C or 5°C for up to 12 months compared to samples stored at -70°C^a

Biomarker ^c , unit	QC pool	Storage condition ^b						
		-70°C Reference	-20°C				5°C	
			3 mo	6 mo	9 mo	12 mo	6 mo	12 mo
<i>Iron status indicators</i>								
<i>FER, ng/mL</i>	Low	20.7	20.8	21.0	20.8	21.0	21.2	21.4
	Medium	73.2	73.8	74.5	74.4	73.6	74.3	74.1
	High	130	131	132	131	132	132	133
<i>sTfR, mg/L</i>	Low	2.47	2.45	2.47	2.47	2.42	2.50	2.53
	Medium	4.33	4.35	4.32	4.28	4.26	4.40	4.33
	High	5.00	5.02	5.00	4.93	4.92	4.98	5.13
<i>Iron, µg/dL</i>	Low	74.6	76.3	71.7	72.1	74.5	78.3	82.3
	Medium	121	125	117	122	121	126	129
	High	173	170	159	160	173	181	191
<i>UIBC, µg/dL</i>	Low	159	162	151	151	156	178	140
	Medium	238	246	228	237	235	246	258
	High	196	194	179	180	193	209	223
<i>CRP^d, mg/L</i>	Low	1.40	1.40	1.41	1.45	1.40	1.37	1.37
	High	12.3	12.3	12.2	12.2	12.2	12.3	12.2
<i>Water-soluble vitamins</i>								
<i>S-FOL, nmol/L</i>	Low	9.29	9.25	8.83	8.74	8.70	<LOD ^e	<LOD
	Medium	27.3	25.5	23.9	23.6	23.6	10.1	5.87
	High	54.8	48.6	47.3	46.9	46.7	22.0	12.5
<i>WB-FOL, nmol/L</i>	Low	190	186	182	179	159	n.s. ^f	n.s.
	Medium	258	267	268	271	269	n.s.	n.s.
	High	406	390	388	408	404	n.s.	n.s.
<i>PLP, nmol/L</i>	Low	9.80	9.36	8.73	7.38	6.33	2.81	0.21
	Medium	35.8	34.0	33.9	31.5	25.9	11.5	3.25
	High	58.8	57.2	54.8	48.0	37.2	16.9	3.47
<i>4-PA, nmol/L</i>	Low	10.1	9.59	9.47	9.53	9.35	9.59	9.79
	Medium	21.2	20.4	20.8	21.2	19.9	21.1	22.3
	High	46.8	47.2	46.0	45.9	47.0	46.7	47.9
<i>B12, pg/mL</i>	Low	301	306	299	303	303	310	311
	Medium	373	379	373	381	383	382	382
	High	550	560	562	566	564	564	575
<i>tHcy, µmol/L</i>	Low	6.12	6.13	6.16	6.04	6.09	6.17	6.36
	Medium	10.9	11.1	10.9	11.1	11.4	10.8	11.9
	High	24.9	25.3	21.0	25.9	25.5	25.2	27.1
<i>MMA, nmol/L</i>	Low	180	n.s.	181	n.s.	182	190	202
	Medium	521	n.s.	511	n.s.	512	514	517
	High	1999	n.s.	2039	n.s.	2016	2014	2162

VIC ^d , mg/dL	Low	0.73	0.53	0.49	0.36	0.28	n.s.	n.s.
	High	1.96	1.78	1.73	1.44	1.31	n.s.	n.s.
<i>Fat-soluble vitamins</i>								
VIA, µg/dL	Low	19.6	19.9	19.6	19.7	20.4	20.5	19.9
	Medium	46.5	46.0	46.1	45.5	45.6	45.0	44.6
	High	71.0	67.6	67.6	67.2	67.1	68.5	68.3
VIE, µg/dL	Low	338	338	340	347	331	334	339
	Medium	776	780	776	779	776	771	754
	High	1518	1506	1508	1509	1507	1495	1472
γ-TOC, µg/dL	Low	74.0	74.4	74.9	75.3	74.7	72.9	75.7
	Medium	258	258	256	257	257	256	255
	High	232	230	229	233	229	229	228
25OHD ₃ , nmol/L	Low	27.8	26.7	25.7	26.6	26.9	27.4	27.2
	Medium	46.7	46.6	45.4	46.5	46.6	48.1	47.8
	High	77.6	76.5	79.6	76.7	78.6	76.6	79.6
25OHD ₂ ^g , nmol/L	Low	1.44	1.86	1.54	1.71	1.68	1.76	1.66
	Medium	5.89	5.00	5.53	5.48	5.30	5.75	5.73
	High	19.4	19.7	20.1	19.8	19.9	20.0	21.3

^a Abbreviations: 25OHD₂, 25-hydroxyvitamin D₂; 25OHD₃, 25-hydroxyvitamin D₃; 4PA, 4-pyridoxic acid; B12, vitamin B-12; CRP, C-reactive protein; FER, ferritin; MMA, methylmalonic acid; PLP, pyridoxal-5'-phosphate; S-FOL, serum folate; sTfR, soluble transferrin receptor; tHcy, total homocysteine; γ-TOC, γ-tocopherol; UIBC, unsaturated iron-binding capacity; VIA, vitamin A (retinol); VIE, vitamin E (α-tocopherol); VIC, vitamin C (ascorbic acid); WB-FOL, whole blood folate

^b Reference samples were stored at -70°C until analysis at study completion (about 12 mo); samples for the -20°C storage conditions were stored at -20°C for 3, 6, 9, and 12 mo and then at -70°C for about 9, 6, 3, and 0 mo until analysis; samples for the 5°C storage conditions were stored at 5°C for 6 and 12 mo and then at -70°C for about 6 and 0 mo until analysis; all conditions were analyzed in the same analytical run; 3 vials and 2 replicates/vial (n=6) were analyzed for the following conditions: reference, 3 mo and 9 mo at -20°C, 6 mo and 12 mo at 5°C; 6 vials and 2 replicates/vial (n=12) were analyzed for the following conditions: 6 mo and 12 mo at -20°C

^c All biomarkers were measured in serum except for whole blood folate

^d Only 2 quality control samples were analyzed

^e LOD for S-FOL was 2 nmol/L

^f n.s., no samples available for analysis

^g LOD for 25OHD₂ was 1.45 nmol/L

Supplemental Table 3. First time point of significant change in biomarker concentration after storage at -20°C and 5°C and acceptability of the change based on biologic variation

Biomarker	Storage at -20°C				Storage at 5°C	
	3 mo	6 mo	9 mo	12 mo	6 mo	12 mo
<i>Iron status indicators</i>						
FER		1.5			1.8	
sTfR		-1.7				
Iron					4.7	
UIBC						
CRP						
<i>Water-soluble vitamins</i>						
S-FOL		-10.5			-61.5	
WB-FOL					n.s.	n.s.
PLP			-18.6		-70.2	
4-PA						
B12						3.3
tHcy						6.2
MMA	n.s.		n.s.			6.5
VIC		-23			n.s.	n.s.
<i>Fat-soluble vitamins</i>						
VIA						
VIE						
γ-TOC						
25OHD ₃						
25OHD ₂						

Numbers indicate the percent change compared to optimum storage at -70°C. Shading indicates acceptability of change: green (optimum bias) and yellow (desirable bias) means acceptable change; pink means unacceptable change. Empty cell means no significant change was observed ($P \geq 0.05$). Abbreviations: 25OHD₂, 25-hydroxyvitamin D₂; 25OHD₃, 25-hydroxyvitamin D₃; 4PA, 4-pyridoxic acid; B12, vitamin B-12; CRP, C-reactive protein; FER, ferritin; MMA, methylmalonic acid; n.s., no samples available for analysis; PLP, pyridoxal-5'-phosphate; S-FOL, serum folate; sTfR, soluble transferrin receptor; tHcy, total homocysteine; γ-TOC, γ-tocopherol; UIBC, unsaturated iron-binding capacity; VIA, vitamin A (retinol); VIE, vitamin E (α-tocopherol); VIC, vitamin C (ascorbic acid); WB-FOL, whole blood folate.