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Stability of HIV-1 RNA in blood during specimen handling and storage prior to amplification by NASBA-QT

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Abstract

The influence of different storage temperatures and anticoagulation conditions on the HIV-1 RNA load as measured by NASBA-QT was examined. Blood specimens from 14 HIV-1 infected individuals were processed within 2 h after collection. The HIV-1 RNA load remained stable for at least 6 months when samples were frozen directly at −70°C in lysis buffer. This lysis buffer fully inactivated the virus. When whole EDTA blood was stored, the HIV-1 RNA load was stable for 72 h at 25°C, but it declined within 24 h at 4°C. The HIV-1 RNA load in whole heparinized blood declined significantly after 24 h at both 4 and 25°C. It was slightly lower (average of 0.18 log ml^{-1}) than in whole EDTA blood. At 4°C, the HIV-RNA load in serum and EDTA-plasma stored with lysis buffer did not decline up to 14 days. At +30°C, however, the load declined significantly after 2 days. Of clinical significance, the mean load in EDTA plasma was 0.5 log ml^{-1} higher than in serum. This difference was patient dependent (range 0.1–0.7 log ml^{-1}). We thus recommend, for quantifying HIV-1 RNA by NASBA, to use preferably EDTA blood which is kept at room temperature until plasma separation. When using heparinized blood, the plasma should be stored frozen within 8 h. © 1997 Elsevier Science B.V.

Keywords: HIV-1 RNA; Stability; Storage conditions; NASBA-QT

1. Introduction

The amount of HIV-1 in a patient's blood has been shown to predict the period to clinical progression, both in natural history studies (Jurriaans

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et al., 1994; Mellors et al., 1996; Bruisten et al., 1997) and in studies where antiviral therapy was given (Saag et al., 1996; Katzenstein et al., 1996). Commercial tests have been developed which measure HIV-1 RNA quantities in blood plasma or serum. These are based on either nucleic acid amplification, such as quantitative PCR (Amplicor HIV-1 Monitor) and quantitative NASBA (NASBA HIV-1 RNA QT) (Mulder et al., 1994; van Gemen et al., 1993), or on signal amplification (bDNA, Quantiplex HIV-1 RNA) (Todd et al., 1995). The accuracy of these tests is dependent on the reproducibility and sensitivity of the amplification procedure, and the choice of the primers (Vandamme et al., 1995), as well as on the efficiency of the isolation of nucleic acids (Verhofstede et al., 1996) and on the handling of the blood samples before the nucleic acid isolation. The last issue has been studied for both HIV-1 and HCV RNA using PCR (Wang et al., 1992; Winters et al., 1993; Cuypers et al., 1992; Busch et al., 1992) and bDNA (Todd et al., 1995; Holodniy et al., 1995) but not for NASBA-QT.

The current study concerns the variation in HIV-1 load measurements using NASBA-QT due to different collection and storage conditions. Some retrospective studies used serum (probably for historical reasons (Holodniy et al., 1991a)), however the viral HCV and HIV-1 RNA load was shown to be lower in serum relative to plasma using PCR (Wang et al., 1992) or bDNA (Holodniy et al., 1995). Heparin, which is also often used as an anticoagulant, (partly) inhibited both the PCR and the bDNA test (Beutler et al., 1990; Holodniy et al., 1991b; Wang et al., 1992; Holodniy et al., 1995). We studied the influence of different anticoagulants on the quantification by NASBA-QT by pairwise testing samples derived from the same HIV-positive individual.

The stability of HIV-1 RNA in whole blood was examined at different storage temperatures (4 and 25°C) and times after collection, mimicking the case where blood is not immediately transported from the site of venipuncture to the laboratory where it is analyzed. We furthermore studied the stability of HIV-1 RNA when HIV-infected plasma or serum was directly added to lysis buffer and stored at three different temperatures (-70, 4 and 30°C) for a few days up to several months. The lysis buffer which was used to isolate the viral RNA contains a chaotropic reagent that, theoretically, will destroy and inactivate all viral particles. Possible remaining infectivity of the virus in lysis buffer was also studied.

2. Materials and methods

2.1. Blood samples for storage experiments

All blood samples were processed within 2 h after venipuncture from proven HIV-1 infected individuals who were not receiving anti-retroviral treatment. We used whole blood without anticoagulant for serum preparation and EDTA and heparin anti-coagulated blood to prepare plasma.

2.2. Storage of whole blood

Twenty ml of EDTA blood and also 20 ml of heparinized blood from each HIV-1 positive individual were drawn. About 6 of 20 ml was directly centrifuged and 200 μl of each plasma was added to 900 μl lysis buffer pH 7.2 (Boom et al., 1990) (see below for formulation) in quadruplicate and stored at -70°C ($t = 0$). The rest of the whole blood was aliquotted in 2-ml portions in six empty, dry glass tubes (Vacutainer, 13 × 75 mm, Becton Dickinson, Franklin Lakes, NJ, USA). Three tubes were stored at each temperature (4 and 25°C) for 6, 24 and 72 h. At the indicated time, one tube for each temperature was centrifuged and duplicate vials of 200 μl plasma in 900 μl lysis buffer were prepared and stored at -70°C.

2.3. Storage in lysis buffer pH 7.2

Twenty ml of whole blood and 20 ml of EDTA blood per HIV-positive individual were used to prepare serum and EDTA plasma. In quadruplicate, 200 μl of serum or plasma was added to 900 μl of lysis buffer pH 7.2 and stored frozen at -70°C ($t = 0$). Serum and EDTA plasma were added in the same ratio to lysis buffer pH 7.2 and the vials were stored at $+4$ and $+30$°C, for both

serum and EDTA plasma. After 1, 2, 7 and 14 days (short-term experiment), two vials were transferred from the indicated temperatures to −70°C storage until analysis (within 30 days). For the long term experiment two vials were removed from the −70°C storage and analyzed at 1, 3, and 6 months.

2.4. Nucleic acid isolation and NASBA-QT

Nucleic acids were isolated using the NASBA HIV-1 RNA QT kit (Organon Teknika, Turnhout, Belgium) based on work described elsewhere (Boom et al., 1990; van Gemen et al., 1993). Briefly, the 1100-μl fractions in lysis buffer are quickly thawed and three calibrator RNAs are added, together with 50 μl of silica, to bind the released nucleic acids. After washing and drying, the nucleic acid was dissolved in 50 μl elution buffer. The isolated RNA was used directly for amplification according to the instructions of the manufacturer. In each run an experimental control sample was included.

2.5. Inactivating capacity of lysis buffer

Two types of lysis buffer were tested for their capacity to inhibit the infectivity of HIV-1, one buffer of pH 6.2 and one pH 7.2 (46 mM Tris–HCl of pH 6.2 or 7.2; 4.7 M guanidine thiocyanate; 1.2% Triton X-100 w/v; 20 mM EDTA). Target cells (H9) were cultured in CM-1 medium (RPMI-1640 medium containing 10% fetal calf serum, 100 IU of penicillin G potassium, 100 μg streptomycin sulfate and 2 mM glutamine). A $HIV\text{-}1_{IIIb}$ stock with estimated 50% tissue culture infective dose per ml ($TCID_{50}$ ml^{-1}) of 8 log was diluted 1×, 10×, 100× and 1000×. Of each dilution, 0.1 ml was spiked in 0.9 ml normal human plasma. In three replicate bottles (A, B and C), 0.1 ml of each spiked plasma was aliquotted. To series A and B, respectively, 0.9 ml of lysis buffer pH 6.2 and pH 7.2 was added. To series C, medium was added (positive control). Mixtures were incubated for 5 min at room temperature and subsequently 9.0 ml of serum-free medium was added. The bottles were centrifuged at 4°C at 35 000 rpm for 1 h in a Beckman centrifuge, type 70 rotor. Pellets were washed once with serum-free medium. Each pellet was suspended in 1.0 ml CM-1 medium. Serial three-fold dilutions on series C were made by adding 1.0 ml to 2.0 ml CM-1 medium. The 3^{-7}–3^{-15} dilutions were tested to determine the virus titer. A volume of 1.0 ml H9 target cells (0.5×10^6) was added to each well of 24-well plates and 0.1 ml of test samples and each dilution of the positive control was added to a replicate of eight wells. Cultures were monitored for a period of 4 weeks for viable cell number, and cytopathic effect (CPE) weekly (Tersmette et al., 1988) and for the expression of p24 antigens on days 14 and 28 by the antigen capture assay (Vironostika HIV-1 Antigen, Organon Teknika Corporation, Durham, USA). Uninfected H9 target cells were cultured, as a negative control.

2.6. Statistics

To test the stability at different temperatures during different storage periods, the Wilcoxon signed rank test was performed (level of significance was 0.05, two-sided). Linear Regression analysis was used to correlate serum and plasma data. Appropriate tests are indicated in the Section 3.

3. Results

3.1. Thermal stability of HIV-1 RNA in whole EDTA or heparinized blood

The stability of HIV-1 RNA was studied in whole blood, mimicking the situation that drawn blood is not immediately sent to the laboratory. Both EDTA and heparinized blood was drawn from six HIV-1 infected individuals. The whole blood was kept at 4°C and 25°C and centrifuged after different time intervals to obtain plasma which was stored with lysis buffer at −70°C until analysis. Each time point was measured in duplicate using NASBA-QT. The mean difference in duplicate measurements was 0.06 log ml^{-1}, and the standard deviation (S.D.) was 0.18 log ml^{-1} in accordance with other reports which used

Table 1
Stability of HIV-1 RNA in whole blood at different temperatures

Blood	Time (h)	Log HIV-1 RNA ml^{-1}			
		4°C		25°C	
		Median (range)	p	Median (range)	p
EDTA	0	5.99 (4.46–6.59)		6.01 (4.39–6.53)	
	6	5.93 (4.53–6.39)	0.027	5.90 (4.55–6.59)	NS
	24	5.87 (4.39–6.26)	0.001	5.93 (4.35–6.56)	NS
	72	5.87 (4.26–6.23)	0.001	5.93 (4.48–6.54)	NS
Heparinized	0	5.94 (4.40–6.44)		5.94 (4.35–6.45)	
	6	5.80 (4.35–6.39)	0.016	5.78 (4.34–6.40)	0.020
	24	5.53 (4.15–6.08)	0.001	5.63 (4.22–6.28)	0.001
	72	5.47 (4.18–6.00)	0.001	5.62 (4.19–6.08)	0.001

EDTA and heparinized blood was drawn from six HIV-infected individuals. The plasma samples were prepared and tested in duplicate after storage at 4 or 25°C for the indicated number of hours by NASBA HIV-1 RNA QT.
Time 0: less than 2 h after venipuncture.
P-value, two-sided, Wilcoxon signed rank test: difference from time 0 is tested.
NS, not significant, $P > 0.05$.

NASBA-QT (Revets et al., 1996; Vandamme et al., 1996; Schuurman et al., 1996). This shows that the reproducibility of NASBA HIV-1 RNA QT is excellent, and that only pre-amplification differences which exceeded 0.36 log ml^{-1} (twice the S.D.) are of clinical importance. The results were analyzed relative to the time that the samples arrived in the laboratory.

The effect of the storage temperature is shown in Table 1. The median HIV-1 RNA load at 4°C was statistically significantly lower after 6 h relative to time 0 in both EDTA blood and in heparinized blood. In both types of blood the decline at 4°C was more pronounced after 24 and 72 hours. At 25°C however, the HIV-1 RNA load remained stable in EDTA blood up to 72 h (Table 1). Storage at 25°C of heparinized blood gave a statistically significant reduction after only 6 h ($p = 0.02$) and a statistically/clinically significant decline after 24 and 72 h. We consider the decline of clinical relevance when it is more than twice the S.D. of duplicates.

The difference in mean HIV-1 RNA load between EDTA and heparinized blood as measured by NASBA-QT can be seen in Fig. 1. The HIV-1 load in heparin samples was significantly lower than in EDTA samples ($p < 0.0001$, Wilcoxon signed rank test) with a mean difference of 0.18 log ml^{-1} (95% confidence interval: 0.15 to 0.21). This difference was also statistically significant when only the data from time 0 were tested ($p = 0.005$, Wilcoxon signed rank test), however, the mean difference at that time was 0.06 log ml^{-1}, less than twice the S.D. and thus not of clinical relevance. The EDTA and heparin measurements were significantly correlated ($r = 0.909$, Spearman correlation coefficient), however, the differences were patient-dependent.

3.2. Thermal stability of HIV-1 RNA in serum or EDTA plasma added to lysis buffer

Both EDTA anticoagulated blood and whole blood were received from eight HIV-1 infected individuals for processing in the laboratory within 2 h after venipuncture. EDTA plasma and serum were prepared and immediately stored in lysis buffer at −70°C. This was measured within 1 week (time 0), and after 1, 3 and 6 months. There was no significant decline in HIV-1 RNA load for either EDTA plasma or serum ($p > 0.05$, Wilcoxon signed rank test), confirming the assumption that the HIV-1 RNA is not degraded at −70°C.

In addition, EDTA plasma and serum were stored in lysis buffer for 1, 2, 7 and 14 days at both 4 and 30°C, and afterwards stored at −70°C. The HIV-1 RNA load was measured batch-wise per individual using NASBA-QT. We observed no significant decline in median HIV-1 RNA load at 4°C for either EDTA plasma or serum stored in lysis buffer (Table 2). At 30°C, however, after only 2 days the HIV-1 RNA load declined significantly for both EDTA plasma and serum.

A striking HIV-RNA load difference was found between EDTA plasma and serum. Of all 164 paired serum and plasma measurements (different individuals, temperatures, time points, duplicates) the serum load was lower than the plasma load in 163 cases ($p < 0.0001$, Wilcoxon signed rank test), on average a difference of 0.5 log ml^{-1} (factor 3). This difference was patient dependent and varied from 0.1 to 0.7 log ml^{-1}. It was also statistically and clinically significant when only the data at time 0 were analyzed, with a mean difference of 0.39 log ml^{-1} ($p < 0.0001$, Wilcoxon signed rank test). The EDTA plasma and serum results were significantly correlated ($r = 0.7219$). In Fig. 2, the stability of the HIV-1 RNA load is shown by plotting the mean HIV-1 RNA load in log ml^{-1} for EDTA plasma and serum.

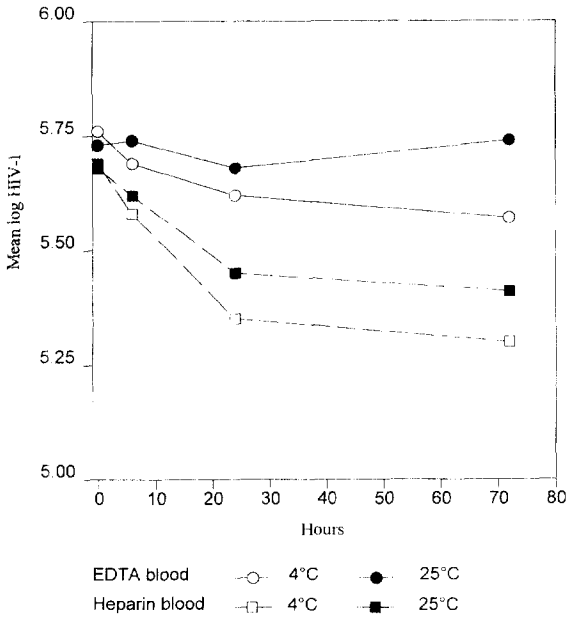

Fig. 1. Stability of HIV-1 RNA in whole blood without lysis buffer. EDTA or heparinized whole blood was stored for the indicated number of hours at either 4 or 25°C, after which plasma was prepared and measured by NASBA-QT. The mean HIV-1 RNA load is expressed in log ml^{-1} copies. Circles indicate EDTA blood, squares for heparinized blood. Open symbols for 4°C, closed symbols for 25°C.

3.3. Inactivating capacity of lysis buffer

For NASBA HIV-1 RNA QT, a lysis buffer is used which mainly contains guanidine thiocyanate, which inhibits degrading enzymes such as RNase, and destroys viral particles. Two types of lysis buffer were tested for inhibition of infectivity, one more acid (pH 6.2) and one neutral buffer (pH 7.2) in a spiking and co-cultivation experiment. The experiment is described in detail in Section 2. Briefly, normal human plasma was spiked with an HIV-1_{IIIb} stock and this was added to lysis buffer 6.2 (series A) or pH 7.2 (series B) or to medium (series C, positive control). These solutions were pelleted and resuspended in culture medium. This was diluted and added to H9 cells to check for the presence of infectious virus. The titer of the 1000-fold diluted HIV-1 stock was 5.1 log $TCID_{50}$ ml^{-1} as calculated on day 28 from both cytophatic effects (CPE) and p24 antigen measurement. The washed pellets from the HIV-1 samples spiked in the lysis buffers (series A and B) all gave abundant growth of the H9 target cells, showing that no inhibition due to this procedure occurred. The p24 antigen assay gave readings below the cut off for all virus stock dilutions in lysis buffers and no CPE were seen at any time in these cultures. We thus conclude that both lysis buffers (pH 6.2 and 7.2) had an inactivating capacity of at least 5.1 log $TCID_{50}$ ml^{-1}.

4. Discussion

Optimal pre-amplification conditions for NASBA HIV-1 RNA QT are the subject of this paper. It was observed that the anticoagulants are of crucial importance for the quantification of HIV-1 sequences. Relative to serum, EDTA

Table 2
Stability of HIV-1 RNA at different temperatures in lysis buffer

Plasma	Time (days)	Log ml^{-1} HIV-1 RNA			
		4°C		30°C	
		Median (range)	p	Median (range)	p
EDTA	0	5.19 (4.70–5.78)		5.19 (4.70–5.78)	
	1	5.23 (4.78–5.74)	NS	5.19 (4.74–5.68)	NS
	2	5.23 (4.74–5.74)	NS	5.07 (4.56–5.68)	0.0009
	7	5.23 (4.74–5.78)	NS	4.45 (4.04–5.18)	0.0001
	14	5.18 (4.74–5.78)	NS	3.59 (3.30–4.33)	0.0001
Serum	0	4.76 (4.27–5.35)		4.76 (4.27–5.35)	
	1	4.78 (4.29–5.36)	NS	4.74 (4.34–5.31)	NS
	2	4.70 (4.31–5.42)	NS	4.68 (3.30–5.34)	0.0008
	7	4.74 (4.35–5.39)	NS	4.14 (3.74–4.74)	0.0001
	14	4.72 (4.45–5.32)	NS	3.30 (3.30–4.00)	0.0001

EDTA plasma and serum samples were prepared from blood of eight HIV-infected individuals. The samples were added to lysis buffer at time 0, and tested in duplicate after storage at 4 or 30°C for the indicated number of days by NASBA HIV-1 RNA QT. Time 0: less than 2 h after venipuncture.
P-value, two-sided, Wilcoxon signed rank test: difference from time 0 is tested.
NS, not significant, $P > 0.05$.

plasma had a greater titer, in agreement with reports using (semi-)quantitative PCR (Winters et al., 1993) and the bDNA assay (Holodniy et al., 1995). This significant difference in HIV-1 RNA load could be measured immediately after the collection of the blood samples (2 h after venipuncture). Two explanations for lower levels in serum can be envisaged. During the coagulation process, viral particles may be trapped in blood clots and thus remain undetected when serum is prepared. Alternatively, proteases and nucleases could be released during the coagulation process, which destroy the virus.

One possible explanation for the difference in HIV-1 RNA load between EDTA and heparinized blood could be that heparin inhibits by 'sticking' to RNA and is less likely to be removed during nucleic acid isolation when blood cells are exposed to heparin for longer periods. Indeed, it was observed that directly after collection, the EDTA-heparin difference was not of clinical relevance, whereas after 24 hours of storage it was. An alternative explanation is that EDTA is a better inhibitor of released proteases and nucleases than heparin. Other studies also described the inhibitory role of heparin on PCR amplification (Beutler et al., 1990; Holodniy et al., 1991b) and on the bDNA assay (Holodniy et al., 1995). In the latter study however, addition of heparin to isolated nucleic acid samples did not inhibit the bDNA assay.

To study what happens to the HIV titer when the time between collection and processing in the laboratory is prolonged, whole blood was stored at either 4 or 25°C. As early as 8 h after venipuncture there was a statistical, but not clinical, significant decline in HIV titer in heparinized blood at both 4 and 25°C. The HIV-1 RNA load in the EDTA blood remained unaffected for 72 h (3 days) when stored at 25°C, however it also significantly declined after 24 h storage at 4°C. The improved stability of HIV-1 RNA in EDTA blood at 25°C versus 4°C was unexpected, since the opposite was found in other studies (Cuypers et al., 1992; Wang et al., 1992). These studies however differed in some respects from our study. Cuypers et al. (1992) and Wang et al. (1992) used (semi-)quantitative RT PCR. Holodniy et al used acid citrate dextrose cell preparation tubes and observed that the HIV-1 sequences were better conserved at 4°C than at room temperature. An explanation for our observation would be that

cells, for example granulocytes, which are kept at 4°C, become activated and release proteases and nucleases which degrade the viral particles. This would be in accordance with the observation by Holodniy et al. (1995), that the HIV-1 sequences were better preserved in separated plasma than in the presence of cells, in experiments using vacutainer tubes. Combining the results of co-cultivation studies one can also conclude that storage of plasma gives a superior preservation of the HIV-1 infectivity than storage of whole blood (Moudgil and Daar, 1993; Vernazza et al., 1993).

Storing EDTA plasma within 24 hours in lysis buffer at −70°C ensures that the titer is stable for at least 6 months. Storage of plasma without lysis buffer preserved the HIV-1 RNA load at −75°C as well (Winters et al., 1993). An advantage of using the lysis buffer is that it fully inactivated the virus, probably because it contains the chaotropic reagent guanidine thiocyanate, which inhibits all proteins and destroys (sub)cellular components. When lysed EDTA plasma was stored at 4°C, the HIV-1 RNA load was not significantly reduced for at least 14 days. However, when left at 30°C the HIV-1 RNA load was significantly declined after only 2 days in EDTA plasma.

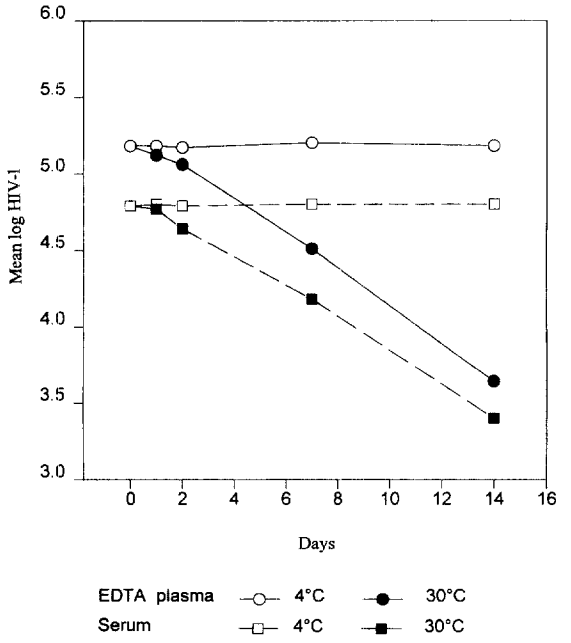

Fig. 2. Stability of HIV-1 RNA in lysis buffer. EDTA plasma or serum in lysis buffer was stored for the indicated number of days at either 4 or 30°C. The HIV-1 RNA load as measured by NASBA-QT is expressed in mean log ml^{-1} copies. Circles indicate EDTA plasma, squares for serum. Open symbols for 4°C, closed symbols for 30°C.

The results of this and other studies suggest the following for measuring accurately the HIV-1 RNA load. Plasma samples should be prepared at room temperature from whole EDTA blood on the day of sampling and stored at −70°C as plasma. It is advisable to ship frozen plasma samples to referral laboratories, since the influences of temperature variations during transportation are hardly predictable. Alternatively, plasma samples can be stored or shipped in lysis buffer at 4°C or −70°C, depending on the time until analysis. Storage in lysis buffer at −20°C is not advised since irreversible coagulation can occur after thawing which leads to problems during nucleic acid isolation (results not shown; see also the instructions of the manufacturer of the NASBA HIV-1 RNA QT kit). When the described procedure is not feasible, the whole EDTA blood samples should be kept at room temperature and arrive in the laboratory within 72 h after venipuncture. Plasma should then be stored frozen at −70°C. Serum produces a lower HIV-1 RNA load compared to EDTA plasma, and is thus not recommended. Heparinized plasma can be used, provided that the plasma is prepared within 8 h after venipuncture (2 h transport and 6 h storage). In any case, the same anticoagulant and transportation temperature and time should be aimed at when longitudinal studies are performed.

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