

Immunoassay to Measure Ataxia-Telangiectasia Mutated Protein in Cellular Lysates

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Background: Ataxia-telangiectasia (A-T) is a neurologic disorder caused by mutations in the ataxia-telangiectasia mutated (ATM) gene. A clinical diagnosis of A-T is confirmed by radiosensitivity testing and immunoblotting for ATM protein. Because both of these tests have long turnaround times (≥ 3 months), we developed a rapid immunoassay to measure ATM protein and determined its sensitivity and specificity for diagnosing A-T.

Methods: Recombinant ATM protein was used for standardization. Lysates of lymphoblastoid cell lines (LCLs) and peripheral blood mononuclear cells (PBMCs) from A-T patients, controls, and A-T heterozygotes were tested for ATM protein by immunoassay.

Results: Between-run imprecision (CV) was $\leq 13\%$. Nuclear lysates from control LCLs and PBMCs had ATM protein concentrations of 49–610 $\mu\text{g/L}$ and 48–943 $\mu\text{g/L}$, respectively. ATM protein was not detectable in LCL nuclear lysates from 18 of 21 A-T patients. The three remaining A-T patients had trace amounts of ATM protein that was confirmed on immunoblots. ATM protein was also detectable in whole-cell lysates from 4×10^6 cells at concentrations of 64–463 $\mu\text{g/L}$ and 42–444 $\mu\text{g/L}$ for control LCLs and PBMCs, respectively. A-T heterozygotes had ATM protein concentrations of 52–98 $\mu\text{g/L}$. ATM protein was stable in PBMCs stored for 1 month at -70°C , but rapidly decreased after 1 day in unprocessed blood.

Conclusions: This ATM protein immunoassay can be used to confirm a diagnosis of A-T in 2 days on small numbers of PBMCs and can potentially identify A-T carriers and individuals at increased risk for cancer.

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Ataxia-telangiectasia (A-T)¹ is a progressive neurodegenerative disorder characterized by a wide range of clinical manifestations, including cerebellar ataxia, conjunctival telangiectases, recurrent sinopulmonary infections, radiosensitivity, and increased cancer susceptibility (1). The onset of unsteadiness and truncal ataxia is usually evident by 1 to 4 years of age. The disease is caused by mutations in the ataxia-telangiectasia mutated (ATM) gene that lead to very little or no ATM protein production in $>95\%$ of A-T patients (2). The majority of mutations cause premature termination codons (3). ATM is a cellular protein with serine-threonine kinase activity that phosphorylates a wide range of substrates critical for cell cycle control, DNA repair, and transcriptional regulation (4).

The clinical diagnosis of A-T in very young infants can be difficult and is often confused with other disorders, such as mild cerebral palsy and Friedreich ataxia. In these very young patients, laboratory testing is extremely helpful. Early diagnosis is also important for genetic counseling, prenatal testing, and family planning. Laboratory tests for A-T include α -fetoprotein, the colony survival assay, and ATM protein detection by immunoblotting (1, 2, 5). Although serum α -fetoprotein concentrations are increased in $\sim 90\%$ of A-T patients, this finding lacks specificity because it is also associated with various malignancies. The colony survival assay detects in vitro hypersensitivity of A-T cells to ionizing radiation (5, 6), but this test also suffers from limited specificity and has a long turnaround time because it requires the production of a lymphoblastoid cell line (LCL) and an incubation period of 10 days to measure radiosensitivity. Similarly, immunoblotting for ATM protein requires a LCL to assure an adequate sample for reliable clinical testing (2).

To improve the diagnostic testing for A-T, we used ATM protein produced in a vaccinia vector to develop an enzyme immunoassay that detects the protein in cell

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¹ Nonstandard abbreviations: A-T, ataxia-telangiectasia; ATM, ataxia-telangiectasia mutated; LCL, lymphoblastoid cell line; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; and BSA, bovine serum albumin.

lysates derived from LCLs and peripheral blood mononuclear cells (PBMCs). ATM protein was readily detectable in cell lysates derived from healthy donors and was undetectable or present in extremely low concentrations in cell lysates from known A-T patients. These findings indicate that this ATM protein enzyme immunoassay is sensitive and can be used for rapid diagnosis of A-T. Furthermore, this assay may have further application for identifying A-T carriers and, possibly, individuals at an increased risk for cancer.

Materials and Methods

SPECIMENS

With informed consent, blood was collected from healthy volunteers into 10-mL glass tubes containing sodium heparin (Becton Dickinson Vacutainer Systems). PBMCs were isolated by centrifugation in a Ficoll-Hypaque density gradient (Amersham Biosciences). Mononuclear cells were counted and used to prepare nuclear lysates or were diluted with phosphate-buffered saline (PBS) containing 10 g/L bovine serum albumin (BSA) and 1 mL/L Tween 20 (Sigma Chemical Co.) and were stored frozen in 0.3-mL aliquots. LCLs were generated from heparinized blood from healthy volunteers (controls) and from blood samples submitted to the clinical laboratory for A-T disease testing. Mononuclear cells were transformed with Epstein-Barr virus and maintained at 37 °C (humidified chamber containing 5% CO₂) in RPMI 1640 (Gibco Invitrogen) containing 150 mL/L heat-inactivated fetal bovine serum (Hyclone) and 10 g/L penicillin/streptomycin (Gibco Invitrogen). This study was reviewed and approved by the Institutional Review Board at UCLA.

STUDY POPULATION

LCLs were randomly selected from 21 A-T patients, 8 obligate A-T heterozygotes (carriers), and 22 healthy donors (controls). The diagnosis of A-T was based on clinical presentation (1), cellular radiosensitivity determined by the colony survival assay (5, 6), and undetectable or low concentrations of ATM protein by immunoblotting (2). ATM gene mutations were also identified for the majority of A-T patients. Heterozygotes were parents of confirmed A-T patients.

GENERATION OF CELLULAR LYSATES

Nuclear lysates from LCLs and PBMCs were prepared by use of NE-PERTM Nuclear and Cytoplasmic Extraction Reagents (Pierce) according to the manufacturer's instructions. The protein concentrations of the nuclear lysates were determined by a modified Bradford method (Bio-Rad Laboratories), and 20–40 µg of nuclear lysate was used for immunoblotting and ATM protein quantification by immunoassay. For whole-cell lysates, LCLs or PBMCs (4×10^6 cells or various numbers as indicated) were aliquoted into 1.5-mL microcentrifuge tubes, centrifuged, and resuspended in 0.3 mL of PBS containing 10 g/L BSA and 1 mL/L Tween 20. Samples were stored at -70 °C

until analysis. Immediately before testing, samples were thawed, vortex-mixed, and sonicated for 3.5 min in a Sonic Dismembrator with a waterbath attachment (Model 550; Fisher Scientific). The Sonic Dismembrator was set to generate ultrasonic energy throughout the waterbath at 20 kHz. After sonication, the samples were centrifuged for 3 min in a quick spin centrifuge at 700g, and the supernatant was analyzed immediately for ATM protein by immunoassay.

IMMUNOBLOTING OF NUCLEAR LYSATES

Western blot analysis of ATM protein has been described previously (2). Briefly, serial twofold dilutions of nuclear lysate (starting at 20 µg of protein) were electrophoresed in a 6% sodium dodecyl sulfate-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane (Bio-Rad), and blocked with 50 g/L nonfat milk. After the membrane was washed, rabbit anti-ATM affinity-purified antibodies at a 1000-fold dilution (Novus Biologicals, Inc.) were added and incubated overnight at 4 °C. After the membrane was blocked and washed, horseradish peroxidase-conjugated donkey anti-rabbit antibodies (immunoglobulin fraction) at a 3000-fold dilution (Amersham) were added and incubated for 35 min at room temperature. An enhanced chemiluminescence detection system (Amersham Biosciences) was used to detect peroxidase activity and was visualized after exposing the blot to Kodak Biomax film for 5 min.

RECOMBINANT ATM PROTEIN

A recombinant vaccinia virus expressing full-length ATM protein tagged with a N-terminal FLAG peptide (DYKD-DDDK) was constructed, as described previously (7). HeLa cells (8×10^6) were infected with the vaccinia virus construct for 32 h. Cells were then lysed and cleared by centrifugation. The lysate was incubated with anti-FLAG M2 affinity resin (Sigma) for 2 h with continuous mixing. The resin was separated by centrifugation, and bound FLAG-ATM protein was eluted by the addition of FLAG peptide (Sigma). The eluted ATM protein was concentrated by use of a Microcon YM-100 centrifugal filter (Millipore Corp.) to ~0.2 g/L. The protein concentration was determined by a modified Bradford method. ATM protein purity was determined by polyacrylamide gel electrophoresis and silver staining and was found to be >90%.

ATM IMMUNOASSAY

Flat-bottomed 96-well high binding EIA/RIA plates (Corning Incorporated) were incubated with two purified mouse monoclonal antibodies, ATM-2C1 (GeneTex) and ATM Ab-8 (Lab Vision Corporation) at 10 mg/L in PBS (pH 7.4) in a final volume of 120 µL. Both antibodies bind the C-terminal region of the ATM protein. A mixture of two monoclonal antibodies was used to capture ATM protein because absorbance readings were higher than when the antibodies were used individually. After 6 h at

room temperature, the plate was washed five times with saline (8.5 g/L NaCl) containing 1 mL/L Tween 20, followed by three washes with deionized water. The plate was then blocked with PBS containing 30 g/L BSA and 1 mL/L Tween 20 for 45 min. Purified ATM protein (serial twofold dilutions starting at 640 $\mu\text{g/L}$) was added in triplicate, and unknown nuclear-cell/whole-cell lysates were added in duplicate. Calibrators and unknown samples were added in a total volume of 120 μL , with PBS containing 10 g/L BSA and 1 mL/L Tween 20 used as diluent. The plate was incubated overnight at room temperature, washed, and blocked; rabbit anti-ATM affinity-purified antibodies (400-fold dilution in 120- μL volume; Novus Biologicals) were then added and incubated for 3 h at room temperature. The plate was washed, blocked, and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (4000-fold dilution; Jackson ImmunoResearch Laboratories, Inc.) for 3 h at room temperature. After the plate was washed, 100 μL of tetramethylbenzidine substrate (1-stepTM Turbo TMB-ELISA; Pierce Biotechnology) was added to each well and incubated for 20–25 min; sulfuric acid (1 mol/L) was then added to stop color formation and produce a yellow color. The absorbance of each well was measured at a wavelength of 450 nm. Background absorbance at 630 nm was subtracted from the absorbance at 450 nm. A calibration curve was generated by use of a linear curve-fitting program with a log-log scale (Microplate Manager Program; Bio-Rad), and ATM concentrations of unknown samples were calculated from the calibration curve.

PRECISION AND STABILITY STUDIES

The intraassay variability of the ATM immunoassay was determined by use of purified recombinant ATM protein. Recombinant ATM protein was diluted in PBS containing 10 g/L BSA and 1 mL/L Tween 20 to arrive at appropriate target concentrations and stored in aliquots at -70°C until testing. The intraassay (within-run) variability (CV) was determined by analyzing ATM protein pools at two different concentrations a total of 10 times in the same microtiter plate. The interassay (between-run) variability was determined by testing two aliquots of each ATM protein pool on different days in a total of nine separate assays. The mean absorbances from duplicate wells were used to calculate 10 (within-run) or 18 (between-run) separate values for each ATM protein pool.

To evaluate the stability of ATM protein in frozen cells, we prepared multiple aliquots of 4×10^6 PBMCs from two healthy donors and stored them frozen at -5 and -70°C . We tested three aliquots, each containing 4×10^6 unfrozen PBMCs, for ATM protein after sonication to determine baseline ATM concentrations. Three aliquots of frozen cells were thawed, sonicated, and assayed for ATM protein (duplicate wells per aliquot) by immunoassay at the indicated times for 2–6 weeks. Values are expressed as the mean (SD).

Results

ATM PROTEIN IMMUNOASSAY

A dose–response curve constructed with serial twofold dilutions of recombinant full-length ATM protein revealed that the ATM protein immunoassay was linear between 640 and 20 $\mu\text{g/L}$, with a correlation coefficient of 0.99 (Fig. 1). Mean values for calibrators (in triplicate) had CVs that were usually $<5\%$. The 20 $\mu\text{g/L}$ calibrator had a mean (SD) absorbance of 0.125 (0.003), and the absorbance was 0.092 (0.002) for the zero calibrator (Fig. 1, dashed line represents the mean + 3 SD). From these values, we conservatively defined the lower limit of detection as 20 $\mu\text{g/L}$. Intraassay imprecision (CV) was $\leq 7.2\%$ at ATM protein concentrations of 108 and 349 $\mu\text{g/L}$ (Table 1). Interassay (between-day) imprecision was $\leq 13\%$ at ATM protein concentrations of 186 and 497 $\mu\text{g/L}$.

The ability of the immunoassay to detect ATM protein in LCL nuclear lysates was examined and compared with the standard method of immunoblotting. As shown in Fig. 2, the ATM protein immunoassay detected ATM protein in LCL nuclear lysates in a dose-dependent fashion, similar to immunoblotting. When compared with immunoblotting, the immunoassay was approximately twofold more sensitive for detecting ATM protein in nuclear cell lysates.

ATM PROTEIN IN NUCLEAR LYSATES FROM CONTROLS AND A-T PATIENTS

LCL nuclear lysates derived from healthy donors (controls) and A-T patients were tested for ATM protein by immunoassay. ATM protein concentrations ranged from 49 to 610 $\mu\text{g/L}$ in nuclear lysates from 20 control LCLs (Fig. 3, left panel). In contrast, ATM protein was undetectable ($<20 \mu\text{g/L}$) in 18 of 21 nuclear lysates from known A-T patients (Fig. 3, middle panel). The remaining

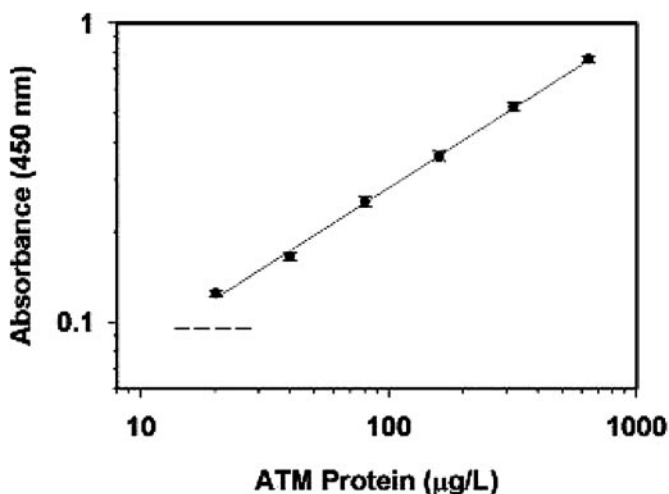


Fig. 1. Dose–response curve for the ATM protein immunoassay.

Serial twofold dilutions of purified recombinant ATM protein were made starting at 640 $\mu\text{g/L}$. Each concentration was assayed in triplicate wells, and mean (SD; error bars) are shown. The dashed line is the mean absorbance reading of the zero calibrator + 3 SD.

Table 1. Precision of the ATM protein enzyme immunoassay.

	n	Mean (SD) ATM, $\mu\text{g/L}$	CV, %
Intraassay ^a	10	108 (6.5)	6.0
	10	349 (25.1)	7.2
Interassay ^b	18	186 (23.9)	13
	18	497 (50.2)	10

^a Recombinant ATM protein was assayed a total of 10 times in the same assay.

^b Recombinant ATM protein was assayed in duplicate in nine assays performed on different days.

three lysates from A-T patients had low but detectable concentrations of ATM protein ranging from 24 to 61 $\mu\text{g/L}$. Interestingly, ATM protein was also detected in these three nuclear lysates from A-T patients by immunoblotting (data not shown). ATM protein was also detectable in nuclear cell lysates from control PBMCs and ranged from 48 to 943 $\mu\text{g/L}$ (Fig. 3, right panel).

ATM PROTEIN IN CELLULAR LYSATES

The preparation of nuclear lysates from LCLs and PBMCs is labor-intensive and occasionally gives variable protein recoveries. Furthermore, to reliably recover nuclear lysates from PBMCs, a minimum of 1×10^7 cells are needed, which can be difficult to obtain from very young children. We therefore investigated the use of whole-cell lysates for measuring ATM protein concentrations by immunoassay. Cell lysates were generated from various numbers of a control LCL and PBMCs by sonication for 3.5 min. Longer sonication times failed to yield higher ATM protein concentrations by immunoassay (data not shown). As shown in Fig. 4, ATM protein was readily detectable in a

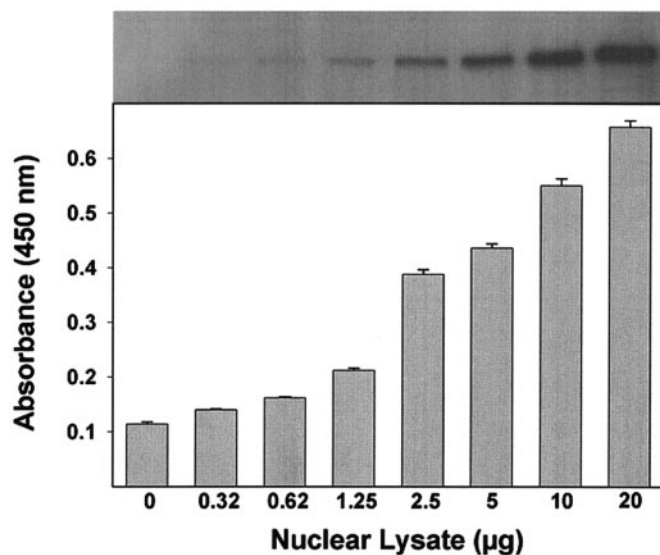


Fig. 2. Comparison of the immunoassay with the standard method of immunoblotting for detection of ATM protein in nuclear lysates.

A LCL nuclear lysate was tested for ATM protein by immunoblotting (top) and immunoassay (bottom). Duplicate wells were tested in the immunoassay and absorbances are expressed as the mean (SD; error bars).

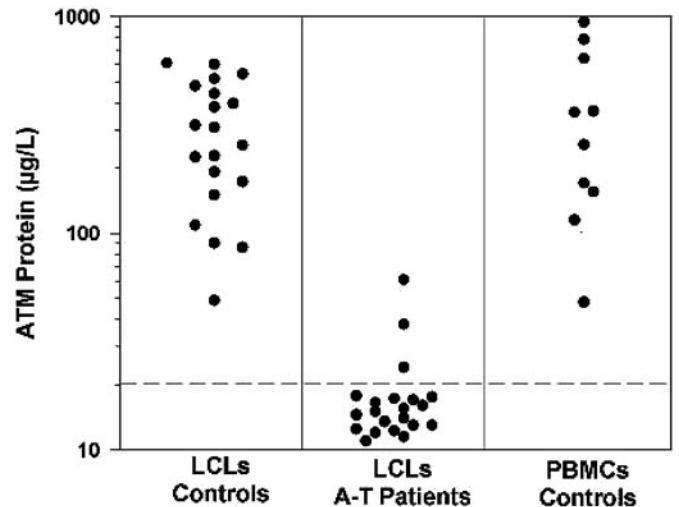


Fig. 3. Quantification of ATM protein by immunoassay in nuclear cell lysates from LCLs and PBMCs.

We tested 40 μg of nuclear cell lysate from 20 control LCLs (left), 21 LCLs from known A-T patients (middle), and 10 control PBMC samples (right) for ATM protein concentrations. The lower limit of detection (20 $\mu\text{g/L}$) is indicated by the dashed line.

LCL and a PBMC whole-cell lysate, and the amount of ATM protein was cell-number-dependent. ATM protein could be detected in as few as 2.5×10^5 cells. The concentration of ATM protein in cell lysates prepared from 4×10^6 cells ranged from 64 to 463 $\mu\text{g/L}$ for 22 control LCLs and from 42 to 444 $\mu\text{g/L}$ for 19 control PBMC preparations (Fig. 5). LCL lysates from eight A-T heterozygotes were also tested and found to contain low but detectable ATM protein concentrations ranging from 52 to 98 $\mu\text{g/L}$ (Fig. 5, right panel)

STABILITY OF ATM PROTEIN

Because PBMCs are often stored frozen before analysis, we investigated the stability of ATM protein in frozen

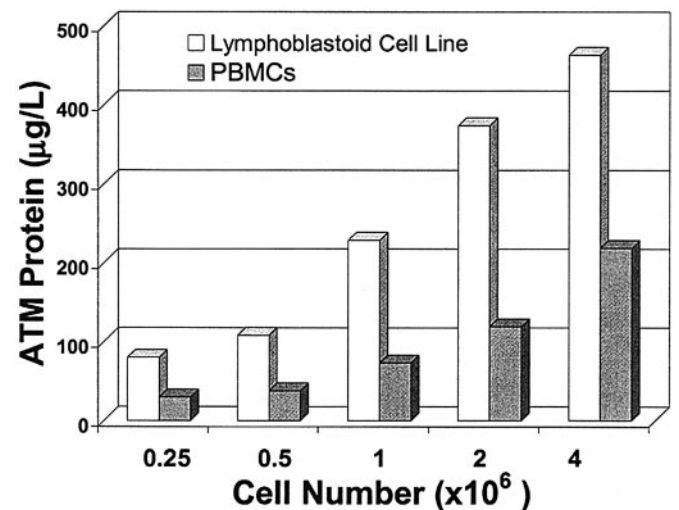


Fig. 4. Detection of ATM protein in whole-cell lysates.

Various numbers of a control LCL and PBMCs were sonicated for 3.5 min and assayed for ATM protein by immunoassay.

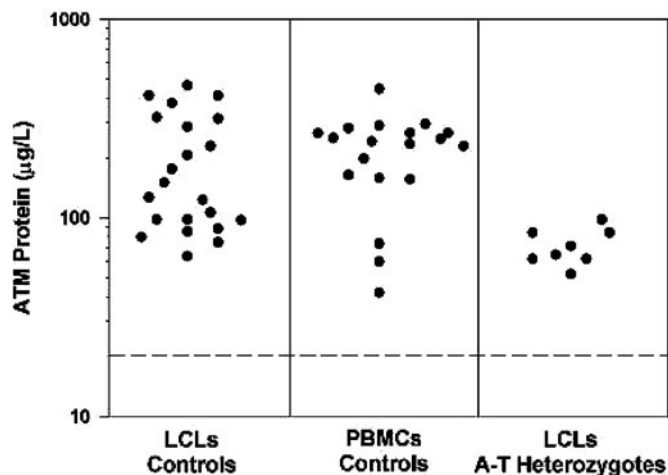


Fig. 5. Quantification of ATM protein in whole-cell lysates of LCLs and PBMCs.

We sonicated and assayed 4×10^6 cells from 22 control LCLs (left), 19 control PBMC samples (middle), and 8 LCLs from A-T heterozygotes (right) for ATM protein. The lower limit of detection ($20 \mu\text{g/L}$) is indicated by the dashed line.

samples. Aliquots containing 4×10^6 control PBMCs from two donors were stored in 0.3 mL of assay buffer at -5 and -70°C and were assayed for ATM protein at various time points. ATM protein in control PBMCs stored at -5°C was stable for only a few days, and the measured concentration decreased by as much as 72% after 1 week of storage (Fig. 6). In contrast, ATM protein was stable in PBMCs stored at -70°C for a 4-week period.

We also examined the stability of ATM protein in heparinized whole blood and found that ATM protein concentrations decrease by 76–85% (three control samples) when stored at room temperature for 1 day (data not

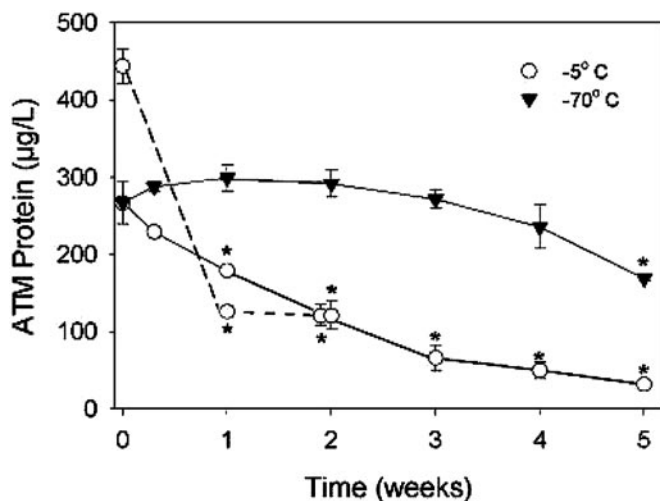


Fig. 6. Stability of ATM protein in PBMCs stored at different freezer temperatures.

Three aliquots of 4×10^6 PBMCs from two control donors were tested at each time point. Cells from one donor were tested after storage at -5°C for only 2 weeks (dashed line). Each data point represents the mean (SD; error bars) of triplicates. * indicates a statistically significant difference compared with time 0 ($P < 0.05$ by a paired *t*-test).

shown). At day 2, ATM protein was undetectable in all three whole-blood samples. The use of other anticoagulants, such as EDTA or ACT, failed to improve the stability of ATM protein in whole-blood samples stored at room temperature (data not shown).

Discussion

Currently, diagnostic laboratory testing for A-T involves demonstrating that lymphoblastoid cells derived from possible A-T patients are hypersensitive to ionizing radiation in a colony survival assay. The colony survival assay is abnormal in almost all A-T patients (>99%), but radiosensitivity can also be observed in cell lines from patients with other DNA repair disorders and in primary immunodeficiencies (5, 6). Because of this, immunoblotting of nuclear lysates for ATM protein is also performed to determine whether ATM protein is being produced. For more than 99% of A-T patients, nuclear lysates from LCLs fail to produce or produce only trace amounts of ATM protein (2). ATM protein concentrations are normal or near normal in other radiosensitive disorders (3). Although the colony survival assay and immunoblotting for ATM protein together have excellent sensitivity (>99%) and specificity (>99%) for diagnosing A-T disease, these tests require the production of a LCL, a labor-intensive process that adds an additional 2–3 months to the testing process. This can be an extremely arduous time for families awaiting a diagnosis. Thus, there is a real need for a rapid, sensitive immunoassay that can detect ATM protein in cellular extracts.

The ATM gene encodes a 370-kDa polypeptide containing 3056 amino acids. The mouse monoclonal antibodies used in our immunoassay to capture ATM protein were generated against a fragment of human ATM protein containing amino acids 2577–3056 (C-terminal region). An ATM fragment containing amino acids 2138–2739 was used to produce rabbit antisera for detection of ATM protein. Thus, in addition to full-length ATM protein, the immunoassay has the potential to detect some forms of truncated ATM protein. However, truncated forms of ATM protein have not been identified in any of the 123 A-T patients who were screened by immunoblot analysis (2).

The ATM protein immunoassay had acceptable within- and between-day analytical imprecision with CVs $\leq 13\%$ at ATM protein concentrations that would be observed for most control cell lysates from 4×10^6 PBMCs. Most cell lysates from A-T patients had ATM protein concentrations below the detection limit of $20 \mu\text{g/L}$, with the exception of three LCL lysates that had low but detectable amounts of ATM protein. Immunoblot analysis was also weakly positive for ATM protein in these three A-T patients. It is of interest that two of these A-T patients producing low amounts of ATM protein have missense mutations in the ATM gene (6188G>A and 8494C>T), which are found in <10% of all A-T patients (1). The

mutations in the third A-T patient have not been identified.

The initial immunoblotting method to detect ATM protein in LCL was developed with whole-cell lysates (8, 9), and it was only recently shown that the sensitivity could be improved by use of nuclear lysates (2). The generation of nuclear cell lysates for immunoblotting is labor-intensive and requires a minimum of 1×10^7 lymphoblastoid cells for adequate protein concentrations. To simplify the procedure and eliminate variable protein recoveries when preparing nuclear lysates, we demonstrated that whole-cell lysates could be used to measure ATM protein by immunoassay. Furthermore, detectable amounts of ATM protein were observed in non-A-T patient lysates derived from PBMCs, eliminating the need for generation of LCLs. Thus, the use of PBMCs in this newly developed immunoassay can dramatically improve testing turnaround times. Although these studies were performed with PBMCs derived from heparinized blood, we also found that EDTA-anticoagulated blood produced comparable ATM protein concentrations with use of nuclear and whole-cell lysates (data not shown).

We were somewhat surprised to discover that ATM protein concentrations in unseparated whole blood decreased rapidly, regardless of the anticoagulant. Blood collection tubes containing protease inhibitors also failed to stabilize whole-blood ATM protein concentrations (data not shown). The instability of ATM protein in whole blood can be a potential problem when unprocessed samples are sent for testing by overnight delivery from remote sites. However, isolated PBMCs were found to be stable for a period of ~1 month when maintained at -70°C , allowing processed samples to be transported long distances for ATM quantification. Although it is unclear why ATM protein is unstable in whole-blood samples, it may be related to DNA repair mechanisms. For example, in lymphoblastoid cells exposed to DNA double-strand break-inducing agents, a large fraction of ATM protein was retained in nuclear aggregates in a nonextractable form (10). The association of ATM with chromatin or subnuclear compartments might block important epitopes on the ATM molecule that are recognized by the antibodies used to capture or detect ATM protein in the immunoassay. It is plausible that storage of whole blood at room temperature leads to oxidative stress and DNA double-strand breaks, with subsequent retention of the ATM pool in nuclear aggregates. Additional studies are currently underway to dissociate ATM protein from nuclear aggregates to improve the stability/detection of ATM protein in whole-blood samples.

As many as one third of A-T patients develop a malignancy, such as lymphoma or leukemia (11). Furthermore, there is a fourfold increase in breast cancer for A-T carriers (heterozygotes) (12–21). This has led to the theory that many breast cancer patients in the general population are A-T carriers (22, 23). However, several recent studies have failed to provide convincing evidence for increased

A-T carrier rates in breast cancer cohorts (24–28). Differences in study design most likely contribute to the conflicting findings among studies. For example, the paucity of truncating ATM mutations in breast cancer patients make protein truncation testing an inefficient screening method (29). The development of a rapid and simple ATM protein immunoassay would be an extremely valuable tool for screening a large cohort of breast cancer patients for A-T heterozygote status. In preliminary studies to address this issue, we tested LCL lysates from eight A-T heterozygotes and found that ATM protein concentrations were lower, on average, than those found in both LCLs and PBMCs from healthy controls. Thus, this immunoassay may be useful for screening potential A-T heterozygotes before expensive DNA sequencing of this large gene.

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