HIV and schistosomiasis : studies in Tanzania

Downs, J.A.

CHAPTER 5

CORRELATION OF SERUM AND DRIED BLOOD SPOT RESULTS FOR QUANTITATION OF SCHISTOSOMA CIRCULATING ANODIC ANTIGEN: A PROOF OF PRINCIPLE

Jennifer A. Downs[a,b], Paul L.A.M. Corstjens[c], Julius Mngara[d], Peter Lutonja[d], Raphael Isingo[d], Mark Urassa[d], Dieuwke Kornelis[e], Govert J. van Dam[e]

[a]Center for Global Health, Weill Cornell Medical College, 402 E. 67th Street, Second Floor, New York, NY, USA; [b]Department of Medicine, Bugando Medical Centre, Box 1370, Mwanza, Tanzania; [c]Department of Molecular Cell Biology, Leiden University Medical Center, Postzone S1-P, Postbus 9600, 2300 RC Leiden, the Netherlands; [d]National Institute for Medical Research – Mwanza Research Centre, Box 1462, Mwanza, Tanzania; [e]Department of Parasitology, Leiden University Medical Center, Leiden, the Netherlands

Acta Trop 2015 Jul 3; 150:59-63.
doi: 10.1016/j.actatropica.2015.06.026

Abstract

Circulating Anodic Antigen (CAA) testing is a powerful, increasingly-used tool for diagnosis of active schistosome infection. We sought to determine the feasibility and reliability of measuring CAA in blood spots collected on Whatman 903 Protein Saver cards, which are the predominant filter papers used worldwide for dried blood spot (DBS) research and clinical care.

CAA was eluted from blood spots collected from 19 individuals onto Whatman 903 cards in Mwanza, Tanzania, and the assay was optimized to achieve CAA ratios comparable to those obtained from the spots' corresponding serum samples. The optimized assay was then used to determine the correlation of serum samples (n=16) with DBS from cards that had been stored for 8 years at ambient temperature. Using a DBS volume equivalent to approximately four times the quantity of serum, CAA testing in DBS had a sensitivity of 76% and a specificity of 79% compared to CAA testing in serum. CAA testing was reliable in samples eluted from Whatman 903 cards that had been stored for 8 years at ambient temperature. The overall kappa coefficient was 0.53 (standard error 0.17, $p<0.001$).

We conclude that CAA can be reliably and accurately measured in DBS collected onto the filter paper that is most commonly used for clinical care and research, and that can be stored from prolonged periods of time. This finding opens new avenues for future work among more than 700 million individuals living in areas worldwide in which schistosomes are endemic.

1. Introduction

The schistosome Circulating Anodic Antigen (CAA) assay is a test of high importance for both the estimated 260 million individuals worldwide with schistosome infection, as well as the 700 million who live in endemic areas and are at risk of schistosome acquisition (1,2). CAA is a glycoprotein produced in the gut of schistosome worms (3) that is secreted into the host bloodstream during active schistosome infection. CAA correlates closely with infection status, making the test useful not only for diagnosis but also for monitoring treatment response (4–7).

Compared to the traditional diagnosis of schistosomiasis by microscopic examination for eggs in multiple urine and stool samples, schistosome antigen testing offers advantages of single sample collection, elimination of labor-intensive work with excrement, and enhanced sensitivity with the potential to detect as little as one worm pair (8). Antigen testing has also been recommended for serologic screening programs in which repeated examinations of urine and stool are logistically not possible (9) and for diagnosis of young children, from whom obtaining urine and stool samples is difficult (10). Given that the CAA assay has recently been developed into a dry-reagent lateral flow assay with a portable reader that can be easily transported to, and operated in, resource-limited settings (11), its utilization will likely continue to increase.

From a laboratory standpoint, the CAA assay is an appealing test. The antigen's unique carbohydrate structure has no known biological equivalent (11,12), and recent modifications make the assay highly sensitive (13). CAA is heat-resistant and extremely stable, remaining detectable in tissue isolated from Egyptian mummies (14). While this would suggest that CAA might be easily measured in dried blood spot (DBS) samples, two early studies that explored this issue in several types of filter paper have shown that CAA was detectable but that available concentrations were low (15,16). Of note, these studies did not evaluate Whatman 903 Protein Saver cards, which cost approximately USD $1.50 each and are the most commonly-used filter papers for HIV testing and monitoring worldwide, including early infant HIV diagnosis and HIV drug resistance genotyping (17,18). Whatman 903 cards have additionally been validated for detection of malaria gametocyte RNA by qRT-PCR (19,20). Given that numerous projects currently collect DBS from regions in which schistosomiasis, HIV, and malaria are co-endemic and that the ability to test these DBS for schistosomiasis would be useful for future patient care and research, we sought to determine the feasibility and reliability of measuring CAA in DBS on Whatman 903 cards, as compared to serum, from patients in Tanzania where all three infections are co-endemic.

2. Materials and Methods

2.1 Study site. Samples for this study were collected in the Kisesa ward in northwest Tanzania, located approximately 20 kilometers east of Mwanza city. We have previously demonstrated that the prevalence of schistosomiasis by CAA in serum is ~50% among adult women in this region near Lake Victoria (21), with a similar prevalence in adult men (unpublished data). Urine and stool microscopy demonstrated that the predominant species in the region is *S. mansoni*, with approximately 25% of community-based participants in prior studies having *S. mansoni* ova visualized in stool and approximately 2-3% having *S. haematobium* ova visualized in urine.

2.2 Sample collection for assay optimization. In April 2012, we invited women of childbearing age who were seeking care for themselves or their children at the Kisesa Health Centre to participate in this study. Four milliliters of blood were collected by venipuncture from the antecubital fossa and five spots of blood (each ~13 millimeters in diameter) were collected by fingerstick lancet onto Whatman 903 Protein Saver cards (GE Healthcare Life Sciences, Piscataway, NJ, USA). Cards were dried away from direct sunlight, placed into individual zip bags 24 hours after collection, and stored at room temperature until processing. Venous blood was centrifuged upon return to the National Institute for Medical Research Laboratory in Mwanza City approximately 20 kilometers away, and serum was stored at -20C. All women were given empiric praziquantel (40mg/kg) in accordance with World Health Organization guidelines (22).

2.2 Dried blood spot sample preparation. To elute dried samples from cards, we cut sections from the DBS and placed them into eppendorfs containing 100μL of phosphate-buffered saline. The sections were incubated overnight at 4C, then placed on a shaker for 1 hour at 37C, after which 100μL of 4% (w/v) tricholoroacetic acid (TCA) was added and the mixture vortexed and centrifuged. We first eluted a 24mm^2 DBS into a total volume of 200μL. In order to increase the sensitivity, we subsequently eluted DBS with a total area of 144mm^2 into final concentration of 2% TCA. The supernatant from these elutions was concentrated to a final volume of 20-30μL using a 10kDa concentration device (Amicon Ultra-0.5ml Centrifugal Filters, Millipore Corp).

2.3 CAA test strip preparation and testing. Serum samples (20μL) were mixed with an equal volume of 4% TCA, vortexed, and centrifuged. Twenty microliters of this supernatant, 20μL of the supernatant of the eluted samples from the small circles, or all of the concentrated eluate supernatants were subsequently mixed with assay buffer containing UCP reporter particles labelled with anti-CAA

McAb, incubated, and applied to CAA-specific lateral flow test strips as previously described (8,11,23). Strips from serum samples were prepared at the local laboratory in Mwanza, Tanzania and then shipped with DBS to Leiden University Medical Centre for subsequent testing. Elutions, CAA strip preparation for eluted samples, and CAA strip scanning using a modified Packard Fluorocount meter were performed at Leiden University Medical Centre. The test line signal was normalized to the control line signal for each individual sample. Standards of known concentrations, run with each assay, were used to construct a standard curve and to determine the cut-off point above which samples were considered positive, which corresponded with 30pg/ml.

2.4 Additional testing in banked dried blood spots. Following optimization of the assay, we obtained additional paired serum and DBS samples from the same Kisesa region that had been collected onto the same Whatman 903 cards from adults aged 15-49 years in 2005. The cards had been stored with desiccant packs in separate zip bags at room temperature. CAA strips from serum samples were prepared in Mwanza; DBS elution and subsequent processing were performed in the Netherlands. Elutions were performed using 216mm^2 of dried sample and 600 µl PBS as described above, with the subsequent addition of 138 µl of 12% TCA (resulting in a final 2% (w/v)). The total volume of supernatant was concentrated to 20-30 µl.

2.5 Statistical analysis. Results were entered into Microsoft Excel and analyzed using Stata/IC Version 13 (College Station, TX, USA). Correlation between log-transformed CAA concentrations was determined using Pearson's correlation coefficient. Agreement between positive and negative test results was assessed using Cohen's kappa coefficient.

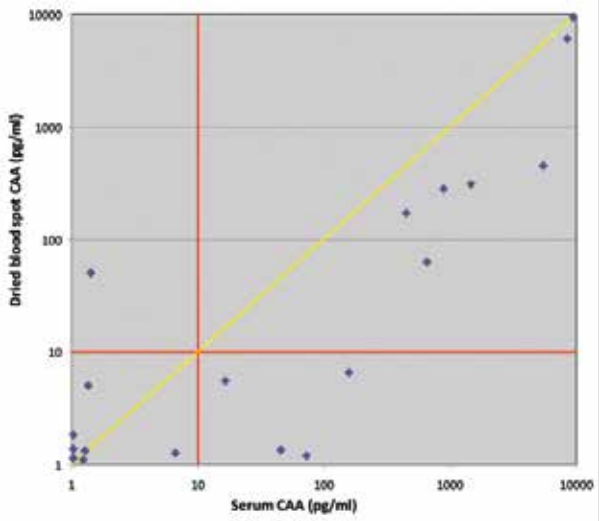
2.6 Ethics. This project was approved by the joint Bugando Medical Centre/ CUHAS Research and Publications Committee, the National Institute for Medical Research in Tanzania, and Weill Cornell Medical College. Study participants provided informed consent for their participation.

3. Results

3.1 Available samples. Paired serum and DBS were available from a total of 35 individuals. Nineteen participants provided fresh blood and DBS in April 2012, which were used for test optimization between April and July 2012. The other 16 patients had both serum and DBScollected and banked in 2005; these samples were tested in March 2013. If serum results from 2005 diverged strongly from DBS results both samples were retested in August 2013.

3.2 Optimization of dried blood spot testing. By serum testing, 11 of the 19 samples (58%) collected for optimization were positive for CAA, with concentrations greater than 10 pg CAA/ml. Testing of DBS was initially performed using small 24mm^2 DBS. This yielded positive results only in four of the samples that had been most strongly CAA-positive on serum testing (CAA >200pg/ml). When the DBS area was subsequently increased to 144mm^2 (corresponding to approximately 30μL of dried serum), 8 of 11 spots were positive (**Figure 1**). Among the 8 serum-negative samples, one was positive for CAA on DBS (50pg/ml, versus 1pg/ml in serum). The remaining seven were negative for CAA both in serum and DBS.

Figure 1. Serum CAA versus optimized dried blood spot values (n=19).

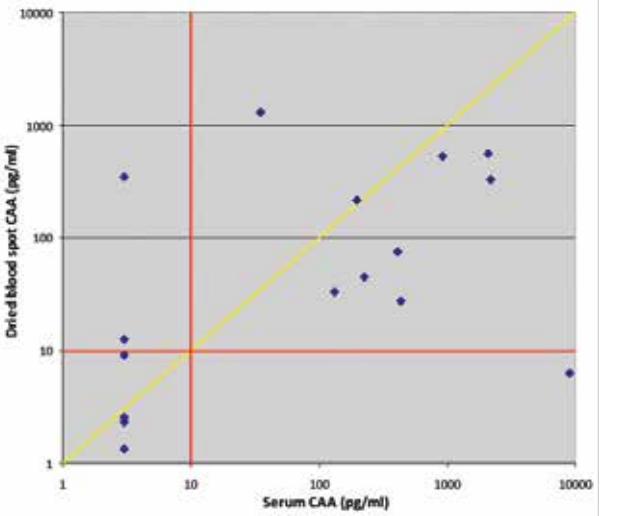


CAA values from serum and from 144mm^2 DBS samples were plotted against each other. Red solid vertical and horizontal lines indicate the cut-off value of 10pg/ml, above which a patient is considered "CAA-positive." The diagonal line indicates perfect 1:1 correlation. The majority of samples' plotted values fall below the diagonal line, indicating higher concentrations obtained from serum than from DBS.

3.3 Application of optimized assay to banked dried blood spots. By serum testing, 10/16 patients (63%) who had serum collected eight years previously were CAA-positive. Their corresponding DBS, which had been stored at room temperature for eight years, were positive in 9 of these 10 patients. **Figure 2** demonstrates the linear correlation between serum and DBS CAA concentrations, with a Pearson's correlation coefficient of 0.70 ($p<0.001$). The three samples that appear most divergent were re-tested blindly and again had similarly divergent values. As seen in the samples used for optimization, CAA concentrations measured in serum appear to be slightly higher than those obtained from the DBS.

CAA values from eight-year-old serum and from DBS samples were plotted against each other. Red solid vertical and horizontal lines indicate the cut-off value of 10pg/ml, above which a patient is considered "CAA-positive." The diagonal line indicates perfect 1:1 correlation. The majority of samples' plotted values fall below the diagonal line, indicating higher concentrations obtained from serum than from blood spots. DBS testing correctly identified as CAA-positive 9 of 10 samples that were CAA-positive in serum.

Figure 2. Serum CAA versus Dried Blood Spot Samples after Eight Years of Storage.



3.4 Overall performance measures. Considering all 35 samples tested with serum as the "gold standard," the overall sensitivity and specificity of DBS testing were 76% (16/21) and 79% (11/14), respectively. The overall kappa coefficient was 0.53 (standard error=0.17, $p<0.001$).

4. Discussion

Our work demonstrates that schistosome CAA can be reliably and accurately quantitated in DBS stored on Whatman 903 Protein Saver cards, yielding close correlation with serum values. Because Whatman 903 cards are the most commonly-used filter paper for DBS collection and preservation in clinical care and research, this finding has important implications for future work among more than 700 million individuals living in areas in which schistosomes are endemic. First, we have validated a simple means by which DBS that have been banked for cohort studies can be tested to determine whether pre-existing schistosomiasis was a risk factor for later patient events, such as subsequent HIV acquisition or development of cancer. In addition, our work demonstrates an opportunity to enhance both patient care and clinical research by coupling schistosome testing with HIV- or malaria-related testing in the same DBS. The ability to test for CAA in DBS represents a streamlined way in which schistosome testing can be

enhanced, either on its own or in concert with other projects, thereby increasing attention to this neglected parasitic infection.

In contrast to a prior report that very little CAA could be detected in DBS stored at room temperature for longer than three months (15), we successfully eluted and quantified CAA in Protein Saver cards that had been stored at room temperature for approximately eight years. Our finding that the CAA sensitivity remained high in eight-year-old spots suggests minimal degradation of CAA during long-term storage at ambient temperatures on these optimized storage papers. While long-term Protein Saver card storage at ambient temperatures has been demonstrated to be suboptimal for subsequent study of nucleic acids (24) and antibodies (25), our findings support the conclusion that this is not an issue for this schistosome-derived glycoprotein that is stable enough to be detected in Egyptian mummies (14).

Our results support and further the findings previously reported for CAA testing in DBS on other paper types—that CAA is indeed detectable in DBS but that it is incompletely eluted. In order to achieve CAA ratios comparable to those obtained from 20 μL of serum, we had to use an area of blood on the Protein Saver card that corresponded to approximately 30 μL of serum. An earlier study similarly reported recovering less than one-third of a known quantity of CAA from several other common filter papers (15). In contrast, 80-100% of the original CAA quantity was obtained from blood dried onto an inert polypropylene fiber web matrix (15). Investigators postulated that poor CAA recovery from filter papers compared to the matrix was related to the papers' higher fiber densities, smaller pore sizes, and the interaction of CAA with reactive hydroxyl and carboxylic acid groups on the hydrophilic surface of the cellulose. Whatman 903 Protein Saver cards are similarly made from nearly pure cellulose, with a comparable pore size and a larger fiber density than papers previously tested (26). Our data shows that these challenges, while not trivial, become surmountable by increasing the area of blood spot used. Our prolonged overnight elution, as compared to 30 minutes in the prior study (15), may also be an important factor in maximizing recovery.

Our study had to deal with some practical limitations. Mainly, due to small sample size and the finite quantity of sample available from DBS, we were not able to test multiple iterations of our elution protocol in order to optimize the performance of CAA testing in DBS. It is likely that the protocol can be further optimized to increase sensitivity (e.g. extended (24-hour) elution time, larger elution-volume to card-surface ratio, elevated temperature and shaking, and use of detergents were not explored to the fullest). We also did not have microscopy data from stool or urine, and therefore designed this study to determine the correlation between

CAA testing in DBS and in serum rather than to compare CAA testing in DBS with parasitological data.

In conclusion, our work demonstrates the feasibility and reliability of CAA testing in DBS collected onto Whatman 903 cards. Such DBS collection is a cost-effective, convenient way to obtain, transport, and store blood samples. Many of the remote resource-poor settings in which the simplicity of DBS collection is most needed are also those plagued by schistosomiasis. Our work opens new avenues for testing these at-risk populations, with the flexibility to test both recent samples for clinical care and older samples, regardless of storage temperature, to explore research questions. Increased implementation of CAA testing has the potential to benefit 700 million people living in schistosome-endemic areas through exploration of new hypotheses related to interactions between schistosomiasis, co-infections, and non-communicable diseases in these vulnerable individuals.

Acknowledgements
The authors wish to thank the staff at Kisesa Health Centre for assisting with this project and the study participants for their enthusiastic participation. We are also grateful to our colleagues at the National Institute for Medical Research – Mwanza Research Centre, and particularly to its Director John Changalucha, for their support. This work was supported in part by the National Institutes of Health (K23 AI110238) and by the University of Georgia Research Foundation, Inc. (Schistosomiasis Consortium for Operational Research and Evaluation [SCORE] project).

References

1. World Health Organization. Schistosomiasis Fact Sheet No 115 [Internet]. 2015 [cited 2015 Sep 4]. Available from: http://www.who.int/topics/schistosomiasis/en/
2. World Health Organization. Schistosomiasis - A major public health problem [Internet]. 2013. Available from: http://www.who.int/schistosomiasis/en/
3. De Water R, Fransen JA, Deelder AM. Ultrastructural localization of the circulating anodic antigen in the digestive tract of Schistosoma mansoni using monoclonal antibodies in an immunogold labeling procedure. Am J Trop Med Hyg. 1986 May;35(3):549–58.
4. Van Dam GJ, Bogitsh BJ, van Zeyl RJ, Rotmans JP, Deelder AM. Schistosoma mansoni: in vitro and in vivo excretion of CAA and CCA by developing schistosomula and adult worms. J Parasitol. 1996 Aug;82(4):557–64.
5. De Jonge N, De Caluwé P, Hilberath GW, Krijger FW, Polderman AM, Deelder AM. Circulating anodic antigen levels in serum before and after

chemotherapy with praziquantel in schistosomiasis mansoni. Trans R Soc Trop Med Hyg. 1989;83(3):368–72.
6. Van Lieshout L, de Jonge N, Bassily S, Mansour MM, Deelder AM. Assessment of cure in schistosomiasis patients after chemotherapy with praziquantel by quantitation of circulating anodic antigen (CAA) in urine. Am J Trop Med Hyg. 1991 Mar;44(3):323–8.
7. Kremsner PG, Enyong P, Krijger FW, De Jonge N, Zotter GM, Thalhammer F, et al. Circulating anodic and cathodic antigen in serum and urine from Schistosoma haematobium-infected Cameroonian children receiving praziquantel: a longitudinal study. Clin Infect Dis. 1994 Mar;18(3):408–13.
8. Corstjens PLAM, De Dood CJ, Kornelis D, Fat EMTK, Wilson RA, Kariuki TM, et al. Tools for diagnosis, monitoring and screening of Schistosoma infections utilizing lateral-flow based assays and upconverting phosphor labels. Parasitology. 2014 Dec;141(14):1841–55.
9. Van Lieshout L, Polderman AM, Deelder AM. Immunodiagnosis of schistosomiasis by determination of the circulating antigens CAA and CCA, in particular in individuals with recent or light infections. Acta Trop. 2000 Oct 23;77(1):69–80.
10. Stothard JR, Sousa-Figueiredo JC de, Sousa-Figuereido JC, Betson M, Adriko M, Arinaitwe M, et al. Schistosoma mansoni Infections in young children: when are schistosome antigens in urine, eggs in stool and antibodies to eggs first detectable? PLoS Negl Trop Dis. 2011 Jan;5(1):e938.
11. Van Dam GJ, de Dood CJ, Lewis M, Deelder AM, van Lieshout L, Tanke HJ, et al. A robust dry reagent lateral flow assay for diagnosis of active schistosomiasis by detection of Schistosoma circulating anodic antigen. Exp Parasitol. 2013 Oct;135(2):274–82.
12. Bergwerff AA, van Dam GJ, Rotmans JP, Deelder AM, Kamerling JP, Vliegenthart JF. The immunologically reactive part of immunopurified circulating anodic antigen from Schistosoma mansoni is a threonine-linked polysaccharide consisting of --> 6)-(beta-D-GlcpA-(1 --> 3))-beta-D-GalpNAc-(1 --> repeating units. J Biol Chem. 1994 Dec 16;269(50):31510–7.
13. Van Dam GJ, Xu J, Bergquist R, de Dood CJ, Utzinger J, Qin Z-Q, et al. An ultra-sensitive assay targeting the circulating anodic antigen for the diagnosis of Schistosoma japonicum in a low-endemic area, People's Republic of China. Acta Trop. 2015 Aug 14;141:190–7.
14. Miller RL, Armelagos GJ, Ikram S, De Jonge N, Krijger FW, Deelder AM. Palaeoepidemiology of Schistosoma infection in mummies. BMJ. 1992 Feb 29;304(6826):555–6.
15. Jamaly S, Chihani T, Deelder AM, Gabone R, Nilsson LA, Ouchterlony O. Polypropylene fibre web, a new matrix for sampling blood for immunodiagnosis of schistosomiasis. Trans R Soc Trop Med Hyg. 1997;91(4):412–5.

16. Nilsson LA, van Dam GJ, Deelder AM, Eriksson B, Gabone RM, Schmeisser S. The fibre-web blood sampling technique applied to serological diagnosis of schistosomiasis mansoni. Trans R Soc Trop Med Hyg. 2001;95(1):33–5.
17. Masciotra S, Khamadi S, Bilé E, Puren A, Fonjungo P, Nguyen S, et al. Evaluation of blood collection filter papers for HIV-1 DNA PCR. J Clin Virol. 2012 Oct;55(2):101–6.
18. Rottinghaus E, Bile E, Modukanele M, Maruping M, Mine M, Nkengasong J, et al. Comparison of Ahlstrom grade 226, Munktell TFN, and Whatman 903 filter papers for dried blood spot specimen collection and subsequent HIV-1 load and drug resistance genotyping analysis. J Clin Microbiol. 2013 Jan;51(1):55–60.
19. Mlambo G, Vasquez Y, LeBlanc R, Sullivan D, Kumar N. A filter paper method for the detection of Plasmodium falciparum gametocytes by reverse transcription polymerase chain reaction. Am J Trop Med Hyg. 2008 Jan;78(1):114–6.
20. Jones S, Sutherland CJ, Hermsen C, Arens T, Teelen K, Hallett R, et al. Filter paper collection of Plasmodium falciparum mRNA for detecting low-density gametocytes. Malar J. 2012 Jan;11:266.
21. Downs JA, van Dam GJ, Changalucha JM, Corstjens PLAM, Peck RN, de Dood CJ, et al. Association of Schistosomiasis and HIV Infection in Tanzania. Am J Trop Med Hyg. 2012 Oct 1;87(5):868–73.
22. World Health Organization. Preventive chemotherapy in human helminthiasis. [Internet]. 2006. Available from: http://www.who.int/neglected_diseases/preventive_chemotherapy/pct_manual/en/
23. Corstjens PL, van Lieshout L, Zuiderwijk M, Kornelis D, Tanke HJ, Deelder AM, et al. Up-converting phosphor technology-based lateral flow assay for detection of Schistosoma circulating anodic antigen in serum. J Clin Microbiol. 2008 Jan;46(1):171–6.
24. McNulty A, Jennings C, Bennett D, Fitzgibbon J, Bremer JW, Ussery M, et al. Evaluation of dried blood spots for human immunodeficiency virus type 1 drug resistance testing. J Clin Microbiol. American Society for Microbiology (ASM); 2007 Feb 1;45(2):517–21.
25. Corran PH, Cook J, Lynch C, Leendertse H, Manjurano A, Griffin J, et al. Dried blood spots as a source of anti-malarial antibodies for epidemiological studies. Malar J. 2008 Jan;7:195.
26. Whatman International Limited. Whatman Product Guide [Internet]. 2007. Available from: http://exiquim.com/descargas/Tratamiento de Muestras/Whatman.pdf