A versatile multidimensional protein purification system with full Internet remote control based on a standard HPLC system

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The standard Äkta Explorer high-performance liquid chromatography (HPLC) system has limitations for the automation of multidimensional protein purification. Here, we describe simple modifications that allow for automated multidimensional purification protocols to extend the possibilities of the Äkta three-dimensional purification kit in terms of column number, flexibility of volumes stocked for re-injection of samples, and available choice of buffers. These modifications do not preclude the use of standard one-dimensional purification protocols. Additionally, we demonstrate a technology for encrypted full remote control of the machine over the Internet by cost-effective use of standard asymmetric digital subscriber line (ADSL) that enables direct remote interaction with the machine without preventing local control. A 4-column purification scheme, including equilibration and cleaning in place (CIP) procedures, was implemented on such a system. It significantly increased reproducibility and shortened processing time by 85%, as compared with manual operation, thus allowing for automated protein purification overnight.

Protein purification protocols, once established, have to be highly reproducible to guarantee the success of subsequent applications such as protein crystallography (1,2). Reproducibility is a decisive issue if a protein is difficult to crystallize, but this is true also for other structural or kinetic studies such as inhibitor screening. The critical factor is not only the degree of purity itself (3), but also the capability to maintain constant quality over extended time periods and with different operators.

To achieve the highest purity, many protein purification protocols involve sequential separation over several chromatographic columns that work according to different separation principles, optimized for a given protein. Such multidimensional protocols start with complex mixtures (mostly crude extracts or homogenates) to end up with a fraction containing the highly enriched protein of interest (4). Although the addition of an affinity tag for heterologously expressed proteins facilitates the purification procedure, several different columns may still be necessary, even when using automated high-throughput systems (2,5–8). Such multidimensional protocols are generally based on biocompatible integrated high-performance liquid chromatography (HPLC) systems, such as the Äkta Explorer (GE Healthcare, Zurich, Switzerland). However, if each column is run separately and operated manually, it is a very time-consuming procedure with the risk of significant batch-to-batch variability due to the human factor. There are commercial solutions to automated multidimensional chromatography, but these have limitations as well. For example, the 3D Kit for Äkta systems only allows for three-dimensional (3-D) purifications, and sample volumes recovered for re-injection between columns are limited. Furthermore, the standard setup only allows connecting up to 11 buffers or solutions, which is insufficient for equilibration, run, and cleaning in place (CIP) of more than three columns.

These system limitations would necessitate manual interventions, which inevitably would lead to time loss while waiting for an operator, and batch-to-batch variability affecting the quality of the final protein preparation. To circumvent these problems, we have modified the system layout to take advantage of a certain flexibility of this system. We report here a fully automated Äkta setup for 4- and higher-dimensional protein purifications with remote control via the Internet. Based on this setup, an exemplary purification protocol is presented that is completed in only 19 h as compared with ~1 week with manual operation, and yields protein of consistently high quality.

System hardware. All products are from GE Healthcare unless mentioned otherwise. An Äkta 100 Explorer Air with a preparative, green tubing kit, including a P960 sample pump, a 10-mm UV/VIS flow cell, and a fraction collector Frac 950 was placed in a chromatography refrigerator (Unichromat 2025; Uniequip, Munich, Germany). The standard setup was modified in 10 places as follows, described in order from buffer preparation to fraction collection (for a detailed connectivity diagram, see Supplementary Figure 1):

1. An additional 8-port valve (Cat. no. IV-908, new UniNet address 8) was installed in front of input B2.0 This allowed for connecting 7 additional buffers (i.e., the solutions to run CIP procedures for all columns sequentially by a method queue) to pump B.

2. A large polycarbonate canister (20-L Nalgene Clearboy; Nalgene/Thermo Fisher Scientific, Rochester, NY, USA) for distilled water was connected via a degasser (Cat. no. VE 7510, Viscotec, Stuttgart, Germany) to inputs B1 and A1.8. The latter allows for flushing of the flow path between V6 and pump A, thus separating incompatible solvents used for CIP (e.g., pure ethanol and 8 M urea). In this setup, the inputs A1.1, A2, B1 and B2 can still be used for the standard "buffer preparation" mode of the Äkta in case of manual runs. In addition, the water canister was connected to the sample valve (V5, position 8) for flushing pump P960.

3. The 5-mL Mixer (Cat. no. M-925) was equipped with a new, self-made

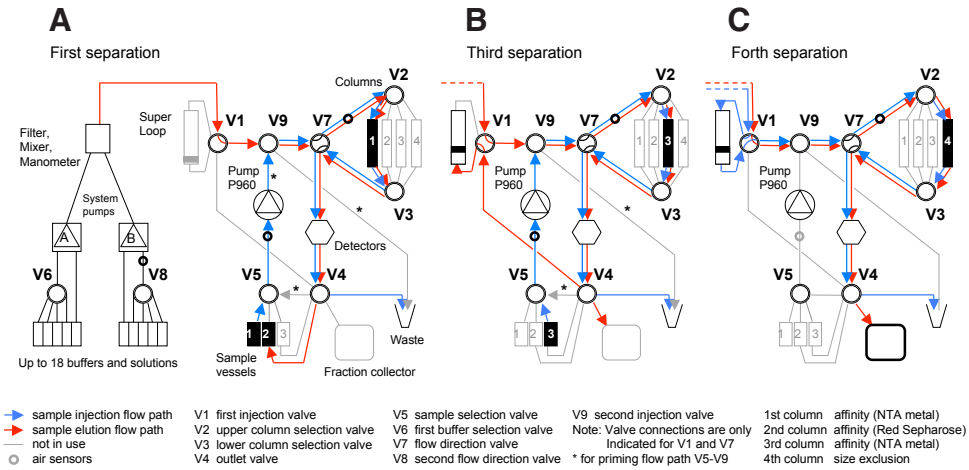

Figure 1. Flow path of a 4-dimensional Äkta setup used to purify AMPK complex. Main tubing connections are given, showing the complete flow path for sample injection (blue arrows) and sample elution (red arrows) of (A) the first chromatography (NTA metal affinity, XK 26/40 column with 140 mL Protino Ni-IDA) and the analogous second chromatography (affinity, XK26/20 column with 45 mL Reactive Red 120 Sepharose), (B) the third chromatography (NTA metal affinity, 2 mL Ni-HP column), and (C) the forth chromatography (size exclusion chromatography, Superdex 200 16/60 column). The flow path for equilibration and CIP is not shown, except for priming the V5-V9 tubing (*), but the necessary tubing is included. Further details are given in Materials and Methods, and a more detailed connectivity diagram is given in Supplementary Figure 1.

magnetic stirrer, containing two strong (N38 magnetization) NdFeB magnets (Cat. no. K-08-C; Supermagnete, Uster, Switzerland) in a glass fiber-armed polytetrafluoroethylene (PTFE) rotor. The latter was cut out of a PTFE block on a lathe with a mortise, and the NdFeB magnets were fixed inside by Araldite epoxy resin (Migros, Thusis, Switzerland) and radially aligned by an external magnetic field until hardening of the resin. This allowed strong and more efficient mixing as necessary for highly viscous buffers (>5 mPa·s) containing, for example, glycerol and/or sucrose for protein stabilization.

4. An additional injection valve (Cat. no. INV-907, new UniNet address 9) was placed between the existing injection (V1) and flow direction valves (V7). This allowed connecting V9 for "direct load" via the sample pump P960, while maintaining V1 for using a Super Loop (Cat.no. 19-7850-01; or any other loop) with the system pumps (see point 8 below).

5. The outlet valve (V4) at position 8 was connected to the sample valve (V5) at position 7. This enabled the system pumps to flush the flow path between V5 and the second injection valve (V9), including the P960, with buffer.

6. A homemade three-way connector (made from 3 standard fingertight fittings and glued together with Araldite epoxy resin) was inserted into the latter tubing and linked to a flow restrictor (4 bar) to work as an overpressure valve. This was necessary to protect the UV/VIS flow cell from exceeding backpressure in case that outlet valve (V4, position 8) would direct flow toward sample valve (V5) without the latter being correctly switched to position 7.

7. A purge valve was mounted between sample valve (V5) and the air sensor (ASP960) in front of the sample pump (P960). This allowed manual filling and cleaning of sample tubing before starting the chromatographic run, an essential procedure for using the air sensor as a detector of completed sample application to stop injection by pump P960.

8. The outlet valve (V4) at position 7 was connected to the first injection valve (V1) at position 3 (normally the fill port for manual operation) to collect an elution peak fraction directly into a Super Loop. This allows more precise re-injection via the system pump, as compared with direct load via pump P960 with its tolerance of ±2 mL. The tubing connector can be easily replaced by a needle-fill port for manual injection and operation of the machine.

9. Outlet valve (V4) positions 3 to 6 were passed into glass vessels to collect large fractions that can be re-injected via the tubing connected to the sample valve (V5) and pump P960.

10. Additional air sensors have been added between valve V8 and input B2 and in front of the upper column valve V2. They prevent the system from sucking air into pump B or pumping air into the column matrices, respectively, by using the watch function of the Unicorn software.

System software. UNICORN 5.01 software was run on a standard Windows XP (SP1) PC connected to a private, physically separated network (labnet) administered by a Debian Linux server. Encrypted remote access to the Äkta and the labnet was provided by running OpenVPN 2.0 (http://openvpn.net) on the server, providing LZO-compressed IP tunneling. An unencrypted real Virtual Network Computing (VNC) server was run on the control PC and VNC clients on the remote computers, allowing for platform-independent access to the HPLC as if sitting in front of the machine (see Supplementary Material for details).

4-D Chromatography implementation. Using the modified Äkta HPLC setup placed into a refrigerator, a four-dimensional (4-D) purification of His_6-tagged heterotrimeric $\alpha_2\beta_2\gamma_1$ AMP activated protein kinase [AMPK (9–11)] was implemented (see Figure 1 for the flow paths, Figure 2 for protein elution profiles, and Figure 1A in Reference 12 for an SDS-PAGE of the final preparation). Protein was heterologously expressed in bacteria as described (12–14). Seventy grams of wet-weight bacterial pellet was lysed in 200 mL lysis buffer (30% glycerol, 0.5 M sucrose, 2 mM $MgCl_2$, and 50 mM HEPES, pH 8.0, at 7°C) by sonication. The lysate was centrifuged at 23,00× *g* for 1 h (Avanti J-20; Beckman Coulter, Roissy, France), the clear supernatant applied to sample vessel 1 and injected by "direct load" (pump P960) onto column 1 [NTA metal affinity chromatography (XK 26/40 column with 140 mL Protino Ni-IDA; Macherey Nagel, Oensingen, Switzerland), Figures 1A and 2A] until air was detected by the air sensor ASP960 in front of the pump. The column was washed with lysis buffer and eluted with 250 mM imidazol into sample vessel 2 (V4, position 4). Prior to elution, pump P960 and the tubing to the second injection valve (V9) were primed with 20 mL buffer (250 mM imidazol) using the system pumps via V9 (position 1), V7 (position 2), V4 (position 8) and V5 (position 7; for flow path, see Supplementary Figure 1). The elution fraction in vessel 2 was then loaded as above onto column 2 [Red Sepharose affinity chromatography (XK26/20 column with 45 mL Reactive Red 120 Sepharose; Sigma-Aldrich, Buchs, Switzerland), Figure 2B], and the column washed with lysis buffer and eluted by an additional 600 mM NaCl into vessel 3. The pump P960 was primed again as before and the elution fraction loaded onto column 3 [NTA metal affinity

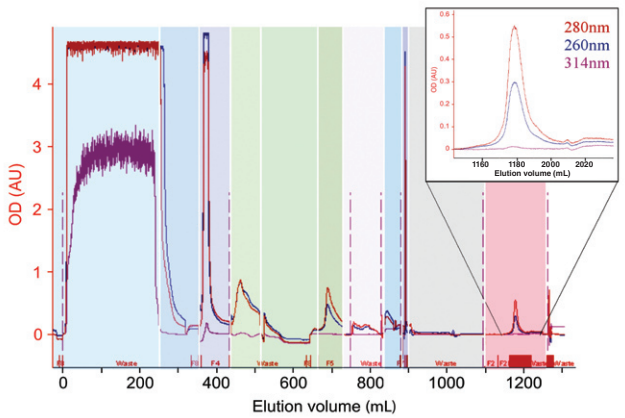

Figure 2. Protein elution profile of a 4-dimensional chromatography run. Purification of $\alpha_2\beta_2\gamma_1$ AMPK on an Äkta setup as shown in Figure 1 (for details, see "System hardware" section.) (A) First chromatography (NTA metal affinity matrix). Light blue, sample loading; dark blue, column washing and flushing the system with elution buffer; violet, elution of the first column into sample vessel 2 (V4, position 4). (B) Second chromatography (Red Sepharose affinity matrix). Light green, loading of the elution of the first column; dark green, column washing; olive, elution into sample vessel 2 (V4, position 5). (C) Third chromatography (NTA metal affinity matrix), necessary for concentration and buffer exchange. Pink, loading of the elution of the second column; blue, column washing; dark blue, concentrated peak eluted into the Super Loop (V4, position 7) connected to the first injection valve. (D) Forth chromatography (size exclusion chromatography). Grey, equilibration of the column while sample is in the Super Loop; red, size exclusion chromatography. The insert shows a magnification of the final elution peak of purified protein. Elution was monitored at 280 nm (protein), 260 nm (nucleotides), and 314 nm.

chromatography (2 mL Ni-HP column; GE Healthcare), Figures 1B and 2C] for concentration and buffer exchange. After a wash with lysis buffer, the column was eluted by 250 mM imidazol, 2 mM tris(2-carboxyethyl)phosphine (TCEP) and 200 mM NaCl. The AMPK elution peak was monitored by automated peak recognition and directed to the fraction collector, except for those 5 mL containing the highest protein concentrations. The latter fraction was directed into the Super Loop (V4, position 7), where it remained in the presence of TCEP to ensure a fully reduced AMPK. Column 4 [size exclusion chromatography (Superdex 200 16/60 column; GE Healthcare), Figures 1C and 2D] was equilibrated with 200 mM NaCl, 2 mM $MgCl_2$, 2 mM TCEP and 50 mM HEPES pH 8.0 at 7°C. Finally, the sample was injected onto column 4, and the AMPK peak was eluted into the fraction collector. The entire purification procedure was carried out at 7°C except for the Superdex column, which was run at 25°C; further experimental details may be found in Reference 12. This protocol, which needed only 18.5 h for full completion, was followed by an automated CIP regeneration procedure. It consisted of serial injections of the following solutions, all separated by an injection of distilled water: 8 M urea and 0.05% SDS (for NTA and Red Sepharose columns), 0.4 M EDTA (pH 7.4) and 0.9 M $NiSO_4$ (additionally for NTA columns), and 0.1 M NaOH (for the Superdex 200 size exclusion column). Following this procedure, no carry-over to subsequent purifications was observed. The purity of the preparation and the lack of carryover was confirmed by Coomassie Blue–stained SDS-PAGE gels of peak fractions, showing the three AMPK subunits as distinct protein bands at the expected molecular weight (see Figure 1A in Reference 12). Quality and reproducibility of this protein preparation were essential for an extended biophysical characterization of the AMPK complex (12). The protocol has been also successfully applied to other AMPK subunit isoform combinations ($\alpha_1\beta_1\gamma_1$, $\alpha_2\beta_1\gamma_1$, and $\alpha_1\beta_2\gamma_1$) (12), as well as isoforms of creatine kinase (unpublished data).

A 4-D purification protocol on an Äkta Explorer system has been successfully set up for automated and remote-controlled operation in a minimum time span, including equilibration and regeneration of all columns (Figures 1 and 2). The standard setup had to be modified and extended to meet the following requirements: (*i*) The availability of at least four consecutive purification steps with recovered and re-injected volumes of unknown size, ranging from milliliters up to several liters, possibly without limitation; (*ii*) the possibility to inject, in the same protocol, fractions of unknown, large size (e.g., bacterial lysate) and fractions of known, precise volume in the milliliter range (e.g., for size exclusion chromatography); (*iii*) overcoming the limitation of connecting only eleven buffers to the system, which is an insufficient number for equilibration, chromatography, and CIP procedures of more than three columns; (*iv*) a technical environment that allows continuous operation of the instrument (24 h/day, 7 days/week) at any temperature between 4°C and 20°C, even when using solutions with high viscosity; and (*v*) implementation of a feasible way for controlling the machine from offsite during purification procedures that last several days.

The commercially available 3D Kit for the Äkta did not offer a solution to these problems. It can only handle three dimensions, the column volumes have to be compatible with each other, and only smaller volumes can be stored in between the runs. However, we found that the problems could be solved by the addition of two valves, a flow restrictor, some additional tubing connections and a Linux PC (Supplementary Figure 1). One additional valve serves as a second buffer/solution selection valve, which not only allows accessing several large vessels (2–20 L) for solvents that are frequently used in automated mode, but also continues to work with the standard built-in "buffer preparation" mode. The other additional valve is used as a second injection valve, which enables the parallel use of "direct load" and Super Loop for sample application in the same protocol, and also an easy set-up for quick one-dimensional runs with manual injection.

CIP procedures to regenerate all columns and to flush the tubing and the Super Loop have been implemented in an analogous, automated way to prevent carry over or contamination in subsequent purifications (see Figure 1A in Reference 12). Stock solutions for CIP (8 M urea, 0.05% SDS, 0.5 M EDTA, 1 M $NiSO_4$, 0.5 M NaOH) and all buffers are supplied by large vessels permanently connected to the system, using the increased number of connectable solutions due to the additional valve V8.

Some additional instruments had to be modified to ensure proper functioning of the entire setup for continuous operation. All instruments were placed inside a refrigerator and checked for their proper functioning down to 4°C. A degasser failed to keep its vacuum at 4°C, so its vacuum pump and valve had to be installed outside the refrigerator. The refrigerator itself has to be operated in a way to avoid local temperature fluctuations that could lead to baseline instability of the UV/VIS signal (likely by the thermal expansion of the fiber optic). Therefore, the built-in air circulation fan of the refrigerator had to be set to permanent function instead of switching on only together with the compressor of the cooling system. Finally, the mixing cell of the Äkta system may fail when using buffers with high viscosity, also resulting in baseline oscillations of the detectors. Already at 20°C, a mixture of 46% glycerol in water exceeds the viscosity limits of the mixer (M-925) (15). An efficient countermeasure was the replacement of

the weak magnetic stirrer by a stronger, homemade one, resulting in efficient mixing of solutions up to 60% glycerol at 4°C.

For a convenient control of the purification progress, remote control via OpenVPN using standard internet connectivity was added. We tested various implementations for the remote access to the machine. However, the one described here was by far the most convenient and in particular, it works reliably and with a bandwidth as slow as 128 Kb/s downstream and 68 Kb/s upstream. The OpenVPN and VNC clients exist for various platforms and the encrypted tunnel can collapse at any time without bringing the Unicorn software on the control PC into a fatal situation. With connections over 10 Mb/s, implementations with the Unicorn-internal remote access capability are also feasible, either by a network drive or by socket over a special port. However, an additional installation of Unicorn on the remote system is necessary, and the flexibility is limited, since the remote system has to connect to the Unicorn server and then blocks local input, making simultaneous remote and local handling practically impossible. With slower connections (e.g., 500 Kb/s downstream and 300 Kb/s upstream with ADSL), remote work with the Unicorn networking installation was very slow or impossible due to internal timeouts, even when using LZO compression. In addition, standard Unicorn-Unicorn connections are not encrypted, so a VPN tunnel has to be built up anyway. Thus, sending the entire graphical output of the control-PC by VNC over a LZO-compressed tunnel proved more practical and much faster than the Unicorn-Unicorn connection.

The automated purification system presented here was used to implement a purification scheme for the heterotrimeric 5′-AMP–activated protein kinase (AMPK) (12) and creatine kinase (unpublished data). AMPK protein was expressed in *E. coli* grown in large bioreactor batch cultures (14). To further decrease batch-to-batch variability, frozen aliquots of bacterial pellet obtained from a single expression were used as starting material. The purification protocol included 4 different columns (12), some of them producing large elution peaks (Figure 2). Manual operation of this purification procedure would take ~1 week (5 working days) and necessitate frequent intervention by an operator, while an automated run took <19 h, with only the initial set-up requiring manual operation. Such a reduction in processing time (by about 85%) has several advantages. First, for example, if one decides to purify a protein from cell lysates in the morning, highly pure protein is available the next day in the afternoon. Furthermore, if the protein is rather unstable in any of the chosen separation conditions, its activity and yield will both be improved during the shorter automated runs, as was the case with the preparation of AMPK.

The automated multidimensional protein purification setup improves the reliability of multidimensional protein purifications as compared with manual handling. It also proved to be an efficient tool for method development. Based on established purification steps, columns and separation conditions of a new step can be designed by storing pre-purified sample in the machine and then using it to perform scouting runs by method queuing. This avoids problems due to variability in pre-purified starting material. The automation is not only convenient, it also allows for a much more time-efficient utilization of the expensive equipment. In our hands, the modified Äkta Explorer setup was in use for more than 95% of time (in a 24 h/day, 7 day/week scheme) over three months without major problems. The full remote accessibility via ADSL saves additional time: for example, a cleaning procedure can be quickly evaluated and repeated if necessary from home in the morning before arriving in the laboratory. Remote control, even from several hundred kilometers away, enables supervision of ongoing work and online briefing collaborators at the local PC in the laboratory.

Given the multiple advantages of the automated HPLC system, the investment in the necessary hardware is modest and the additional software freely available. Most importantly, the advantages and benefits of the modifications are significantly increased productivity and reproducible results in protein purification.

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References

1. **Chapman, T.** 2005. Protein purification: pure but not simple. Nature *434*:795-798.
2. **Stromberg, P., J. Rotticci-Mulder, R. Bjornestedt, and S.R. Schmidt.** 2005. Preparative parallel protein purification (P4). J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. *818*:11-18.
3. **Caylor, C.L., I. Dobrianov, S.G. Lemay, C. Kimmer, S. Kriminski, K.D. Finkelstein, W. Zipfel, W.W. Webb, et al.** 1999. Macromolecular impurities and disorder in protein crystals. Proteins *36*:270-281.
4. **Berkowitz, S.A.** 1989. Protein purification by multidimensional liquid chromatography. Adv. Chromatogr. *29*:175-219.
5. **Scheich, C., V. Sievert, and K. Bussow.** 2003. An automated method for high-throughput protein purification applied to a comparison of His-tag and GST-tag affinity chromatography. BMC Biotechnol. *3*:12.
6. **Opiteck, G.J., S.M. Ramirez, J.W. Jorgenson, and M.A. Moseley 3rd.** 1998. Comprehensive two-dimensional high-performance liquid chromatography for the isolation of overexpressed proteins and proteome mapping. Anal. Biochem. *258*:349-361.
7. **Sigrell, J.A., P. Eklund, M. Galin, L. Hedkvist, P. Liljedahl, C.M. Johansson, T. Pless, and K. Torstenson. 2003. Automated multi-dimen**sional purification of tagged proteins. J. Struct. Funct. Genomics *4*:109-114.
8. **Steen, J., M. Uhlen, S. Hober, and J. Ottosson.** 2006. High-throughput protein purification using an automated set-up for high-yield affinity chromatography. Protein Expr. Purif. *46*:173-178.
9. **Hardie, D.G.** 2007. AMP-activated protein kinase as a drug target. Annu. Rev. Pharmacol. Toxicol. *47*:185-210.
10. **Hardie, D.G. and D. Carling.** 1997. The AMP-activated protein kinase--fuel gauge of the mammalian cell? Eur. J. Biochem. *246*:259-273.
11. **Hardie, D.G., S.A. Hawley, and J.W. Scott.** 2006. AMP-activated protein kinase--development of the energy sensor concept. J. Physiol. *574*:7-15.
12. **Riek, U., R. Scholz, P. Konarev, A. Rufer, M. Suter, A. Nazabal, P. Ringler, M. Chami, et al.** 2008. Structural properties of AMP-activated protein kinase. Dimerization, molecular shape, and changes upon ligand binding. J. Biol. Chem. *283*:18331-18343.
13. **Suter, M., U. Riek, R. Tuerk, U. Schlattner, T. Wallimann, and D. Neumann.** 2006. Dissecting the role of 5′-AMP for allosteric stimulation, activation and deactivation of AMP-activated protein kinase. J. Biol. Chem. *281*:32207-32216.
14. **Riek, U., R. Türk, T. Wallimann, U. Schlattner, and D. Neumann.** 2008. A home-built low cost bioreactor for large-scale bacterial expression of proteins in *E. coli*. Biotechniques *45*:187-189.
15. **Lide, D.R., ed.** 2006. CRC Handbook of Chemistry and Physics, 87th ed., CRC Press, Boca Raton, FL.

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