

Using the NISTmAb reference standard to demonstrate a simple approach to charge variant analysis

Authors

Silvia Millán, Amy Farrell, and Jonathan Bones
Characterisation and Comparability Laboratory,
NIBRT – The National Institute for Bioprocessing Research and Training, Dublin, Ireland

Keywords

NIBRT, Biopharmaceutical, Bioproduction, QA/QC, Biotherapeutic, IgG, Monoclonal antibody (mAb), Critical quality attribute, Intact Protein Analysis, NISTmAb, Reference Material 8671, Humanized IgG1K, Ion Exchange Chromatography, MAbPac SCX-10, ProPac WCX-10, pH Gradient Buffer, CX-1 pH Buffer, Vanquish Flex UHPLC

Application Benefit

- Integrated solution for superior charge variant separation and characterization
- Rapid, easy-to-optimize and highly reproducible method development

Goal

To demonstrate the effectiveness of a simple pH gradient/ion-exchange chromatography workflow approach to the characterization of charge variants in monoclonal antibodies using the NISTmAb reference standard as a model analyte. To show the assay is simple, reproducible, easily optimized, and resolves variants more effectively than previously published salt gradients.¹

Introduction

The characterization of monoclonal antibodies (mAbs) during biopharmaceutical development involves the identification, monitoring, and analysis of charge variants. Antibodies can exhibit changes in charge heterogeneity during production and purification caused by amino acid substitutions, glycosylation, and other post-translational or chemical modifications. Not only can these changes impact stability and activity, they can also cause adverse immunological reactions. Identification of charge

variants in development, and their monitoring throughout manufacturing is therefore critical.^{2,3}

Ion-exchange chromatography (IEX) is widely used for the characterization of therapeutic proteins^{4,5,6} and can be considered a powerful reference technique for the qualitative and quantitative evaluation of charge heterogeneity. IEX separates charge variants by differential interactions with a charged support.

Numerous variants are commonly observed when mAbs are analysed by charged-based separation techniques. These variants are generally referred to as acidic or basic species, compared with the main isoform, and are defined based on their retention times relative to the main peak. Acidic species are variants with lower pI that elute before the main peak by cation exchange (CEX), and basic species are variants with higher pI that elute after the main peak by CEX. Deamidation of asparagine residues and sialic acid content have been widely reported to contribute to the formation of acidic species.⁷ Other modifications have also been shown to result in the generation of acidic species such as the non-classical disulfide linkages or high mannose oligosaccharides content. So far, basic species can be fully explained by known modifications including N-terminal glutamine, N-terminal leader sequences, C-terminal lysine, C-terminal amidation, or succinimide.⁸

State-of-the-art and emerging analytical and biophysical methodologies provide very detailed process and product information; however, their accuracy, precision, robustness, and suitability are also of critical importance. The NIST monoclonal antibody IgG1k (NISTmAb) is intended to provide a well-characterized, longitudinally available test material that is expected to greatly facilitate analytical development applications associated with the characterization of originator and follow-on biologics for the foreseeable future.⁹

The NISTmAb reference material is a recombinant humanized IgG1k expressed in murine suspension culture, which has undergone biopharmaceutical industry standard upstream and downstream purification to remove process related impurities. It is an approximately 150 kDa homodimer of two light chain and two heavy chain subunits linked through both inter- and intra-chain disulfide bonds. The molecule has a high abundance of N-terminal pyroglutamination, C-terminal lysine clipping, and glycosylation of the heavy chain

sub-units. The protein also has low abundance post-translational modifications including methionine oxidation, deamidation, and glycation.⁹

This application note presents the benefits of using pH gradient elution for separation of charge isoforms of NISTmAb reference material 8671 (Lot No. 14HB-D-002) using the combination of the Thermo Scientific™ MAbPac™ SCX-10 strong cation exchange (SCX) column and the Thermo Scientific™ CX-1 pH Gradient Buffers on a Thermo Scientific™ Vanquish™ Flex UHPLC for optimum performance. Additionally, NISTmAb charge variants were characterized following the chromatographic conditions described in the recently published book '*State-of-the art and emerging technologies for therapeutic mAb characterization. Volume 2*', which serves as both a foundational body of NISTmAb product knowledge as well as an evaluation of its suitability as an industry-appropriate reference material (RM), containing representative methods and associated data for the NISTmAb of which extent and quality is comparable to that in a Biologics Licence Application (BLA).¹

Experimental

Chemicals and reagents

- Deionized (DI) water, 18.2 MΩ·cm resistivity
- CX-1 pH Gradient Buffer A, pH 5.6 (P/N 085346)
- CX-1 pH Gradient Buffer B, pH 10.2 (P/N 085348)
- Thermo Scientific™ Chromacol™ Ultra High Recovery vials (P/N 1.2-UHRSV)
- Thermo Scientific™ 9mm Open Top Short Screw AVCS Caps and Septa (P/N 9-SCK(B)-ST1X)
- Carboxypeptidase B (150 units/mg; Roche® Diagnostic P/N 10103233001)
- Fisher Scientific™ Sodium phosphate monobasic monohydrate (P/N BP330-500)
- Sodium phosphate dibasic heptahydrate (P/N BP331-500)
- Sodium chloride (P/N S/3160/60)

Equipment

Vanquish Flex Quaternary UHPLC system, including:

- System Base Vanquish Flex (P/N VF-S01-A)
- Quaternary Pump (P/N VF-P20-A)

- Column Compartment H (P/N VH-C10-A)
- Split Sampler FT (P/N VF-A10-A) with 25 μ L (V=50 μ L) sample loop
- Diode Array Detector HL (P/N VH-D10-A) with Thermo Scientific™ LightPipe™ 10 mm Standard Flow Cell (P/N 6083.0100)

MABPac SCX-10 column, 10 μ m, 4.0 \times 250 mm (P/N 074625)

Thermo Scientific™ ProPac™ WCX-10 column, 10 μ m, 4.0 \times 250 mm (P/N 054993)

Buffers preparation for MABPac SCX analysis

- Pump Eluent A: Dilute CX-1 buffer A pH 5.6 ten times with DI water.
- Pump Eluent B: Dilute CX-1 buffer B pH 10.2 ten times with DI water.

Buffers preparation for ProPac WCX analysis

- Pump Eluent A (20 mM phosphate buffer, pH 6.7): Dissolve 1.2 g of NaH_2PO_4 in approximately 450 mL of DI water. Add 13.449 g NaCl. Titrate to pH 6.7 with monovalent strong base or acid base as needed. Make up to 500 mL with DI water.
- Pump Eluent B (20 mM phosphate buffer, pH 6.7, 0.5 M NaCl): Dissolve 1.2 g of NaH_2PO_4 in approximately 450 mL of DI water. Add 13.449 g NaCl. Titrate to pH 6.7 with monovalent strong base or acid base as needed. Make up to 500 mL with DI water.

Sample preparation

Samples were injected directly at 10 mg/mL in sample buffer (12.5 mmol/L L-histidine, 12.5 mmol/L L-histidine HCl (pH 6.0)). To verify which peaks correspond to C terminal lysine content, samples were digested with carboxypeptidase B (Roche Diagnostic) by incubation at 37 °C for 2 hours at 500 rpm.

UHPLC-UV charge variants analysis using MABPac SCX-10 column

Charge variants were separated by strong cation-exchange chromatography on a Vanquish Flex Quaternary UHPLC system using an optimized linear gradient of 30–80% eluent B (CX-1 Buffer B pH 10.2) at 1 mL/min in 30 min on a MABPac SCX-10 column, 10 μ m, 4.0 \times 250 mm at 30 °C. The detection wavelength was 280 nm. NISTmAb samples were injected in 5 μ L sample buffer.

UHPLC-UV charge variants analysis using ProPac WCX-10 column

Charge variants were separated by weak cation-exchange chromatography¹ on a Vanquish Flex Quaternary UHPLC system at 1 mL/min in 40 min. Eluent A was 20 mM phosphate buffer, pH 6.7, and eluent B was 20 mM phosphate buffer, pH 6.7, with 0.5 M NaCl. The column temperature was 35 °C. The column was held at an initial condition of 3% eluent B for 3 minutes, followed by a linear gradient to 23% eluent B over 30 minutes. The column was held at 23% eluent B for 0.1 minute, ramped up to 99% eluent B over 0.5 minutes, held for 3 minutes, ramped back down to the initial conditions over 0.5 minutes, and then equilibrated at the initial conditions for 3 minutes. The detection wavelength was 280 nm. Injection volume for the NISTmAb samples, dissolved in sample buffer, was 5 μ L.

Data processing and software

Chromatographic software: Thermo Scientific™ Chromeleon™ CDS 7.2 SR4

Results and discussion

NISTmAb charge variants were characterized using a MABPac SCX-10 column (4.0 \times 250 mm, 10 μ m) and a Vanquish Flex system equipped with a quaternary pump and DAD detection.

A pH gradient mode was used for mAb variants separation where the ionic strength of the mobile phase was kept low and constant, while the pH was varied to generate a linear gradient. This buffer system consisted of a low-pH buffer A at pH 5.6 and a high-pH buffer B at pH 10.2. A linear pH gradient from pH 5.6 to 10.2 was generated over the time by running a linear pump gradient from 100 % eluent A to 100 % eluent B (Figure 1a). To optimize the NISTmAb variants separation, a shallower gradient was run (Figure 1b). Figure 1 demonstrates the MABPac SCX-10 10 μ m column providing fast, monoclonal antibody variant analysis using pH-based gradient. Higher resolution was achieved with a shallower gradient of 30% to 80% buffer B (Figure 1b) showing nine variant forms, with the main isoform assigned for peak 5. It illustrates good resolution of C-terminal lysine truncation variants (Figure 1b: peaks 5, 6, and 9), and other acidic and basic variants.

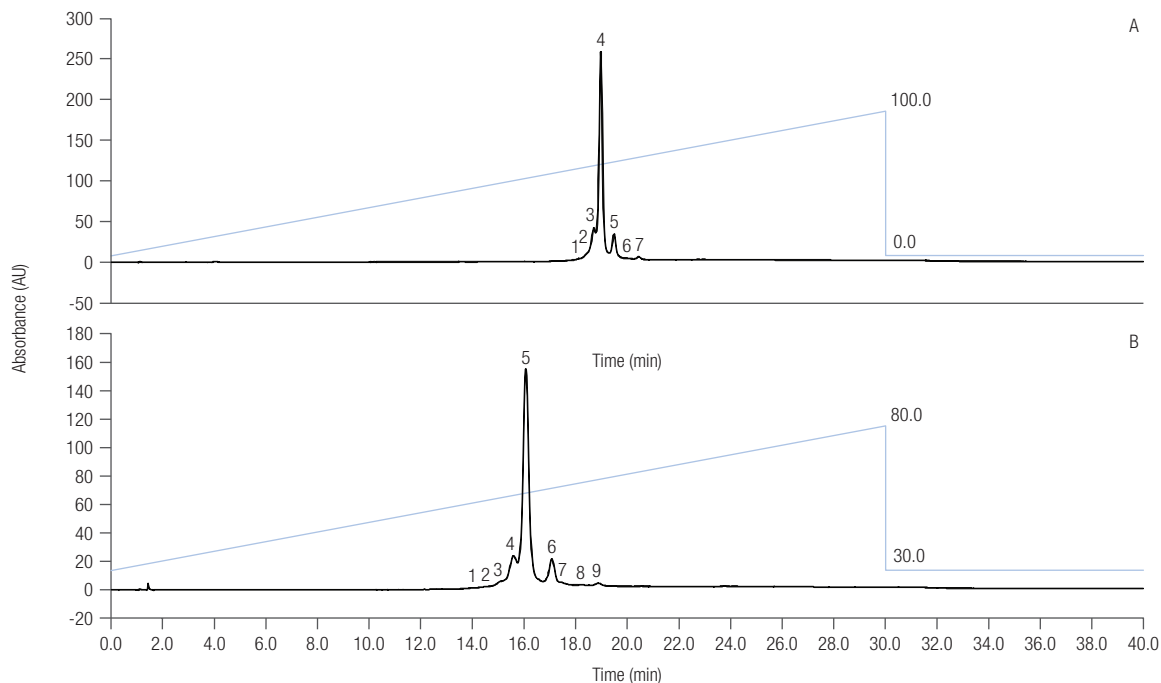


Figure 1. Chromatographic separation of NISTmAb (RM8671) charge variants using pH gradient (A) 0–100% over 30 min and (B) 30–80% B over 30 min, on a MAbPac SCX-10, 10 μ m, 4 \times 250 mm column.

Retention time precision (expressed as relative standard deviation, RSD) is essential in charge variants analysis for peak identification. Retention time precision was measured for repeated injections of the NISTmAb reference sample (Figure 2). Nine

peaks were evaluated. The retention time precision for these peaks is shown in Table 1. The retention time precision for this separation was $\leq 0.25\%$ for all peaks observed.

Table 1. Retention time (RT) precision (n=8) for charge variants peaks from NISTmAb sample RM 8671) shown in Figure 2.

Charge Variant Peak	1	2	3	4	5	6	7	8	9
Minimum RT (min.)	14.17	14.66	15.16	15.58	16.06	17.06	17.44	18.22	18.87
Maximum RT (min.)	14.23	14.78	15.28	15.60	16.08	17.08	17.47	18.25	18.90
Average RT (min.)	14.21	14.71	15.23	15.59	16.07	17.08	17.45	18.24	18.88
RT precision (% RSD)	0.19%	0.25%	0.22%	0.05%	0.05%	0.04%	0.06%	0.07%	0.06%

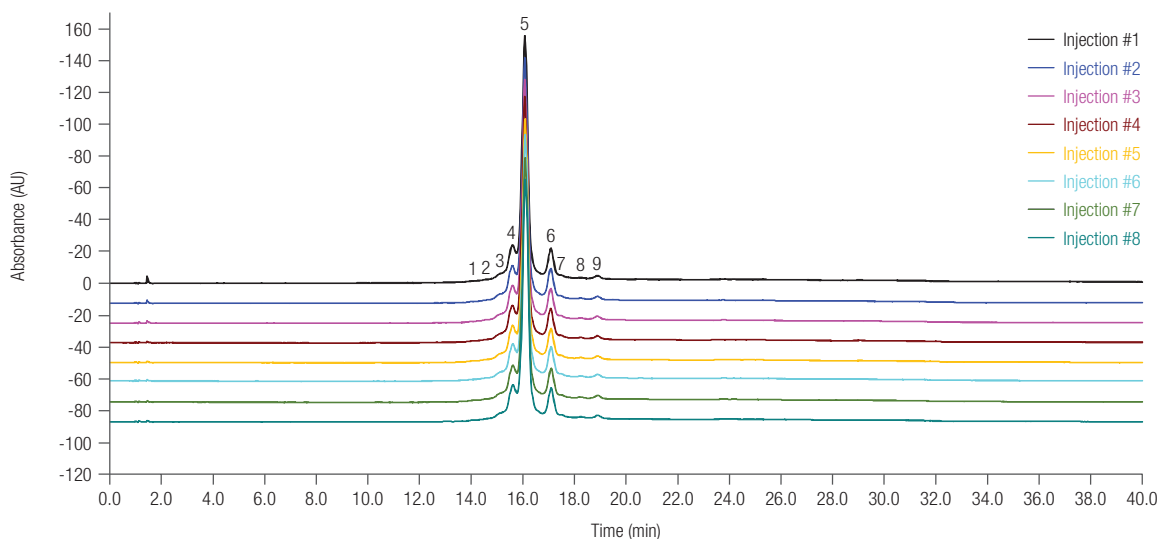


Figure 2. Repeated injections of NISTmAb sample (RM 8671) on a MAbPac SCX-10 column (4.0 \times 250 mm, 10 μ m) and pH-based gradient.

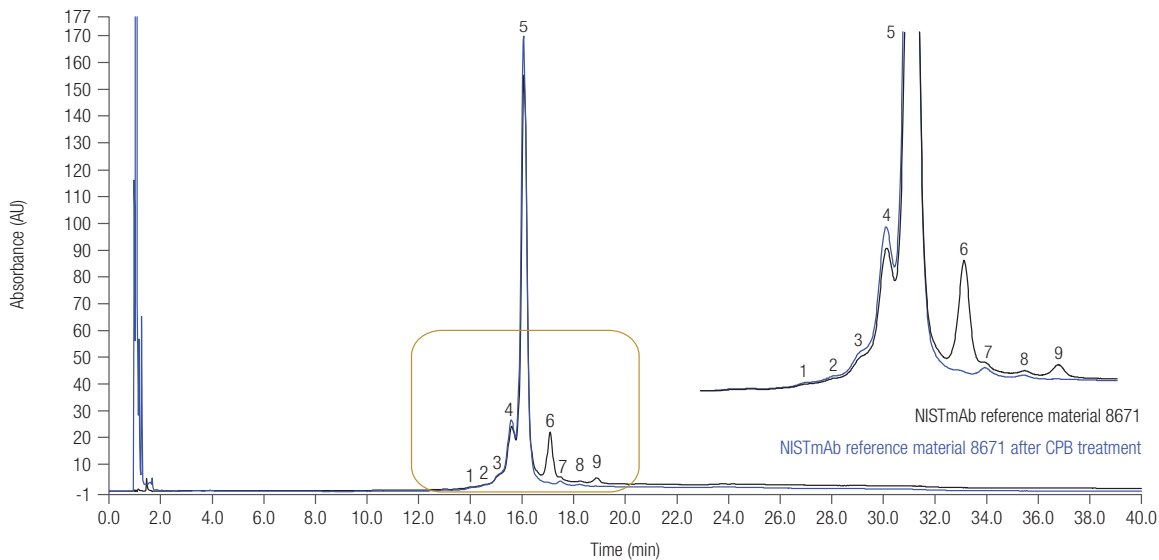


Figure 3. Characterization of NISTmAb (RM 8671) C-terminal lysine truncation variants using MAbPac SCX-10 column (4.0 × 250 mm, 10 μm) and pH gradient mode.

With the aim to verify heavy chain C-terminal lysine content, NISTmAb was treated with carboxypeptidase B (CPB), an exopeptidase that specifically cleaves C terminal lysine residues (Figure 3). This resulted in the absence of peaks 6 and 9 (containing 1 and 2 terminal lysine residues, respectively, on their heavy chains). The decreased peak areas in peaks 6 and 9 are accompanied by a corresponding increase in area of peaks 4 and 5 where no lysine is present (Figure 3, blue trace).

Figure 3 illustrates that three major peaks (black trace: peaks 5, 6, and 9) are due to variations in C-terminal content, after the treatment with CPB only one major peak remains (blue trace: peak 5).

Additionally, NISTmAb charge variants were characterized using a ProPac WCX-10 column as described in detailed in the NISTmAb biopharmaceutical characterization book chapter published by Michels, DA et al.¹ Figure 4 illustrates the comparison of charge variant profiles obtained with the ProPac WCX-10 column using a gradient of phosphate buffer (black trace) and the MAbPac SCX-10 column using a pH gradient mode (blue trace). The MAbPac SCX-10 column provided better resolution and the identification of nine variant forms compared to the results obtained with the ProPac WCX-10 column where only seven variant forms could be detected.

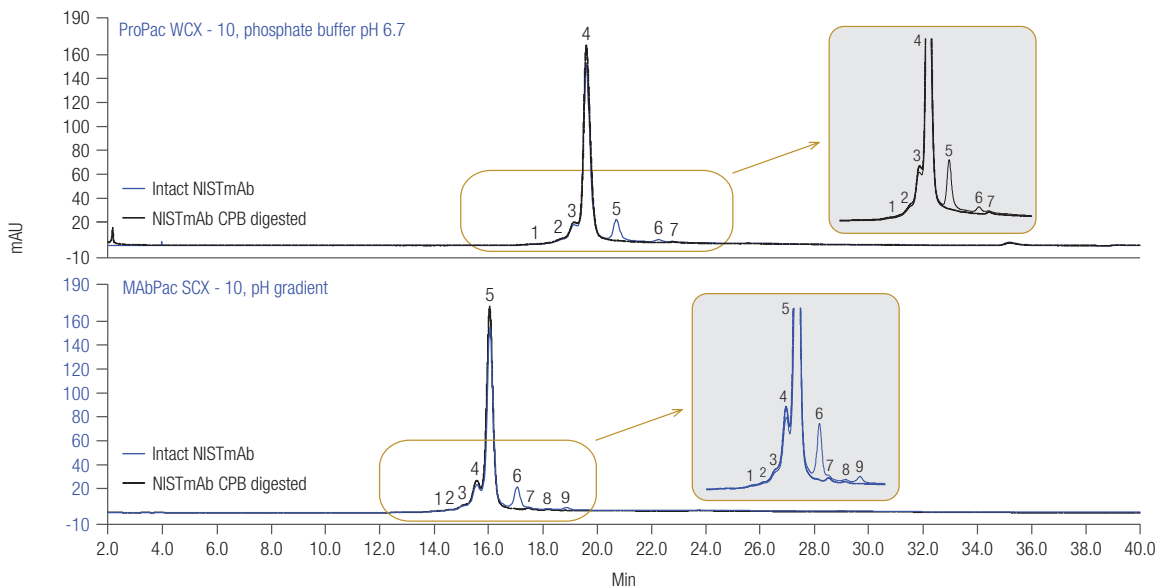


Figure 4. Characterization of NISTmAb (RM 8671) C-terminal lysine truncation variants using a ProPac WCX-10 column (4.0 × 250 mm, 10 μm) and a phosphate buffer gradient (black trace), and a MAbPac SCX-10 column (4.0 × 250 mm, 10 μm) and pH gradient mode (blue trace).

Conclusions

- pH-based gradients can be used for high resolution separation of monoclonal antibody charge variants. The CX-1 buffers are easy to prepare, highly reproducible, and provide a linear pH gradient, which minimize the issues of manually prepared buffers and online mixing variability. The CX-1 pH gradient buffers meet the fast and robust platform method requirements and the gradients can easily be altered to improve resolution.
- The availability of a commercial IgG1k reference material is intended to provide a widely available test product that is not associated with product-specific intellectual property concerns. This material will be useful for the assessment of current and emerging analytical technology and will establish a more robust framework for method quantification.
- The Vanquish Flex platform equipped with the Vanquish quaternary pump and Vanquish DAD detector is a powerful tool for charge variant analysis. Hence, this system can be effectively used for qualitative and quantitative evaluation of charge heterogeneity of NISTmAb reference material facilitating process development for novel mAbs and biosimilar products.
- The MAbPac SCX-10 column is complementary to the ProPac WCX-10 column for monoclonal antibody variant analysis, offering an alternative selectivity and providing higher resolution and efficiency for variant analysis of mAbs samples.
- The MAbPac SCX-10 column provides excellent separation for charge variants from NISTmAb reference material 8671, with excellent retention time precision.

References

1. Michels, D.A.; Ip, A.Y.; Dillo, T.M.; Brorson, K.; Lute, S.; Chavez, B.; Prentice, K.M.; Brady, L.J. and Miller, K.J. Separation Methods and Orthogonal Techniques (book chapter). *State-of-the-Art and Emerging Technologies for Therapeutic mAb Characterization Volume 2. Biopharmaceutical Characterization: The NISTmAb case study*. American Chemical Society, Chapter 5, 236-284, 2015.
2. Zhao, Y.Y.; Wang, N.; Liu, W.H. et al. Charge Variants of an Avastin Biosimilar Isolation, Characterization, In vitro Properties and Pharmacokinetics in Rat. *PLoS ONE*, **2016**, *11* (3), 1-13.
3. Hintersteiner, B; Lingg, N; Janzek, E. et al. Microheterogeneity of therapeutic monoclonal antibodies is governed by changes in the surface charge of the protein. *Biotechnology Journal*, **2016**, *11*, 1617-1627.
4. Zhang, T.; Bourret, J.; Cano, T. Isolation and characterization of therapeutic antibody charge variants using cation exchange displacement chromatography. *Journal of Chromatography A*, **2011**, *1218*, 5079-5086.
5. Teshima, G.; Li, M.X.; Danishmand, R. et al. Separation of oxidized variants of monoclonal antibody by anion-exchange. *Journal of Chromatography A*, **2011**, *1218*, 2091-2097.
6. Fekete, S.; Beck, A.; Veuthey, J.L. et al. Ion-exchange chromatography for the characterization of biopharmaceuticals. *Journal of Pharmaceutical and Biomedical Analysis*, **2015**, *113*, 43-55.
7. Khawli, L.A.; Goswami S.; Hutchinson, R.; Kwong, Z.W.; Yang, J.; Wang X. et al. Charge variants in IgG1: Isolation, characterization, in vitro binding properties and pharmacokinetics in rats. *MAbs*, **2010**, *2*, 613-624.
8. Du, Y.; Walsh, A.; Ehrick, R. et al. Chromatographic analysis of the acidic and basic species of recombinant monoclonal antibodies. *MAbs*, **2012**, *4:5*, 578-585.
9. Tarlov, M.J.; Choquette, S.J. Reference Material 8671. NIST Report of Investigation, October **2016**.

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