



Human SET domain bifurcated 1 (SETDB1), Tudor domain



A Target Enabling Package (TEP)

Gene ID / UniProt ID / EC	9869 / Q15047 / 2.1.1.43
Target Nominator	SGC Internal Nomination
SGC Authors	Mader P, Harding R, Dong A, Tempel W, Walker JR, Dobrovetsky E, Ferreira de Freitas R, Kennedy S, Jurkowska RZ, Vedadi M, Von Delft F, Jeltsch A, Min J, Schapira M, Brown P, Arrowsmith CH, Santhakumar V
Collaborating Authors	N/A
Target PI	Jinrong Min (SGC Toronto)
Therapeutic Area(s)	Oncology
Disease Relevance	SETDB1 amplification is a recurrent driver event in lung cancer
Date approved by TEP Evaluation Group	17 th June 2016
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Affiliations	N/A

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SUMMARY OF PROJECT

SETDB1 is a H3K9 methyltransferase that features a triple Tudor domain, is recurrently amplified in lung cancer, and its depletion reduces cancer growth *in vitro* and *in vivo*. No inhibitor was reported to date. Here we present crystal structures of 5 compounds in complex with the Tudor domain of SETDB1 with associated binding assays by HSQC NMR, SPR and ITC. These compounds should be optimized into a chemical probe to be tested against *SETDB1* amplified cancer cells.

SCIENTIFIC BACKGROUND

SETDB1 is a H3K9 methyltransferase involved in transcriptional silencing with a catalytic SET domain and a triple Tudor domain containing a methyl-lysine binding site. SGC Toronto previously solved the apo structure of the Tudor domain (PDB code [3DLM](#)). Amplification of *SETDB1* in over 15% lung adenocarcinoma correlates with high mRNA and protein levels and its depletion in *SETDB1*-amplified cells reduces cancer growth in cell culture and nude mice models, whereas its overexpression increases tumour invasiveness (1)(2). Several histone methyltransferases are known to have non-catalytic functions that might be alternative targeting strategies. For instance, recognition of H3K9 methylation by the ankyrin repeat of the methyltransferase GLP is required for efficient establishment of H3K9 methylation (3). No catalytic domain inhibitor of SETDB1 has been reported to date. The goal of this TEP is to enable the discovery of potent, selective compounds targeting the Tudor domain of SETDB1.

RESULTS – THE TEP

Proteins purified

SETDB1 Tudor Protein (used for crystallography, ITC, FP)

The expression construct for N-terminal His₆-tagged SETDB1 Tudor domain (residues 197 – 403) subcloned into pET28a-MHL vector was transformed into BL21(DE3)-pRARE2 cells.

¹⁵N SETDB1 Tudor Protein (used for ¹H-¹⁵N TROSY NMR)

The expression construct for N-terminal His₆-tagged SETDB1 Tudor domain (residues 197 – 403) subcloned into pET28a-MHL vector was transformed into BL21(DE3)-pRARE2 cells. The cultures were grown in auto-induction M9 minimal medium containing 1.2 g/l ¹⁵N-ammonium chloride (Sigma 299251)

Biotinylated SETDB1 Tudor Protein (used for SPR)

The expression construct was N-terminally biotinylated SETDB1 Tudor domain (residues 197 – 403) subcloned into pET28BIOH_LIC vector.

Purification of BIOH-SETDB1 (ABC029:G05) used for SPR experiments

The expression construct was N-terminally biotinylated SETDB1 Tudor domain (residues 197 – 403) subcloned into pET28BIOH_LIC vector

Structural data

Apo structure of SETDB1 Tudor domain is published (pdb: [3DLM](#))

Structure with the peptide (from our collaborator) binding to the putative histone binding groove is available and published (4).

5 fragment crystal structures (1.57-1.96 Å) representing both pockets in the putative peptide binding groove are presented and deposited in the PDB.

2 additional fragment crystal structures developed by academic collaborator deposited in the PDB (6au2, 6au3). A manuscript describing the development of fragment hits by academic collaborator is in progress and expected to be submitted by June 2018.

In vitro assays

SPR Assay: Biotinylated SETDB1 Tudor Domain captured onto an SA Chip. Kd of the most active fragment is about 5 millimolar

ITC Assay : Kd of the most active fragment is about 3 millimolar

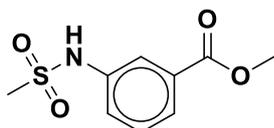
HSQC NMR Assay (qualitative) with ¹⁵N labelled protein. Correlates well with SPR assay

FP assay: FITC labelled H3K9me2K14ac (1-25) peptide was used. None of the fragments are active by FP assay. The unlabelled H3K9me2K14ac (1-25) displaces the FITC-H3K9me2K14ac (1-25) with a Kdisp of 0.89 μM.

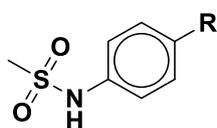
Chemical starting points

3 different chemical series representing both pockets of SETDB1 Tudor domain are presented. Each chemical series have at least 2 examples. Crystal structures presented for at least one example from each series and at least one example from each series shows significant shifts in HSQC NMR. One additional chemical series which show millimolar activity in SPR and ITC and strong shifts in HSQC NMR is being followed up by one academic collaborator (structures not disclosed)

Sulfonamides



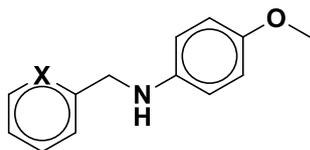
SETDB1-x337 (MRT10241245a)
HSQC NMR : Weak shifts
Crystal structure: 5KH6



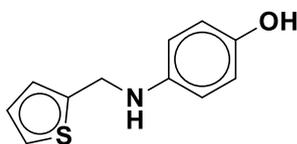
SETDB1-x207 (R=Cl, MRT10241251a)
HSQC NMR : No significant shifts
Crystal structure: 5KCO

Analogue R = OH (MRT10241253a)
HSQC NMR : Weak shifts

Arylalkyl amines

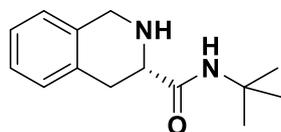


SETDB1-x200 (X=N)
HSQC NMR : Not tested
Crystal structure: 5KCH

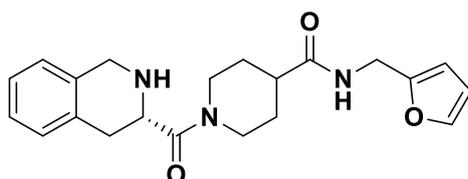


SETDB1-x287 (MRT10241258a)
HSQC NMR : Weak shifts

Tetrahydroisoquinoline Carboxamides



XST00006472a
HSQC NMR: No significant shifts
Crystal structure: 5KE2



MRT00000181a
HSQC NMR: Weak shifts
Crystal structure: 5KE3

IMPORTANT: Please note that the existence of small molecules within this TEP may indicate that chemical matter can bind to the protein in a functionally relevant pocket. As such these molecules should not be used as tools for functional studies of the protein unless otherwise stated as they are not sufficiently potent or well-characterised to be used in cellular studies. The small molecule ligands are intended to be used as the basis for future chemistry optimisation to increase potency and selectivity and yield a chemical probe or lead series.

Future work

- Grow and/or link fragments to increase affinity
- Generate a chemical probe and test whether it selectively kills SETDB1-amplified cancer cells

Collaborations

- Chemistry partners: Andrei Yudin, University of Toronto and Stephen Frye, University of North Carolina
- Other SETDB1 cell assays (Matt Lorincz, University of British Columbia and Yoichi Shinkai, RIKEN)
- Biochemistry: Albert Jeltsch, Stuttgart University

CONCLUSION

SETDB1 is amplified and or overexpressed in cancer patients and depletion in SETDB1-amplified cells reduces cancer growth in cell culture and nude mice models, whereas its overexpression increases tumour invasiveness.

Two academic collaborations have already been working on the optimisation of the fragment hits to develop chemical probes for SETDB1. Crystal structures of the fragment hits representing multiple series of compounds would enable structure based optimisation. Potent and SETDB1 selective chemical probe will help to uncover the potential of SETDB1 inhibitors in cancer.

TEP IMPACT

Publications arising from this work:

A manuscript describing the identification and binding modes of the first small molecule fragment hits that bind to histone peptide binding groove of the TTD of SETDB1 and the biophysical characterization of key compounds was submitted.

- Pavel Mader, Rodrigo Mendoza-Sanchez, Aman Iqbal, Aiping Dong, Elena Dobrovetsky, Victoria Corless, Sean Liew, Scott Houliston, Renato Ferreira De Freitas, David Smil, Carlo Dela Sena, Steven Kennedy, Diego Diaz, Hong Wu, Ludmila Dombrovski, Abdellah Allali-Hassani, Jinrong Min, Matthieu Schapira, Masoud Vedadi, Peter Brown, Vijayaratnam Santhakumar, Andrei Yudin, Cheryl Arrowsmith. (2019) [Identification and Characterization of the First Fragment Hits for SETDB1 Tudor Domain](#). BioRxiv.

FUNDING INFORMATION

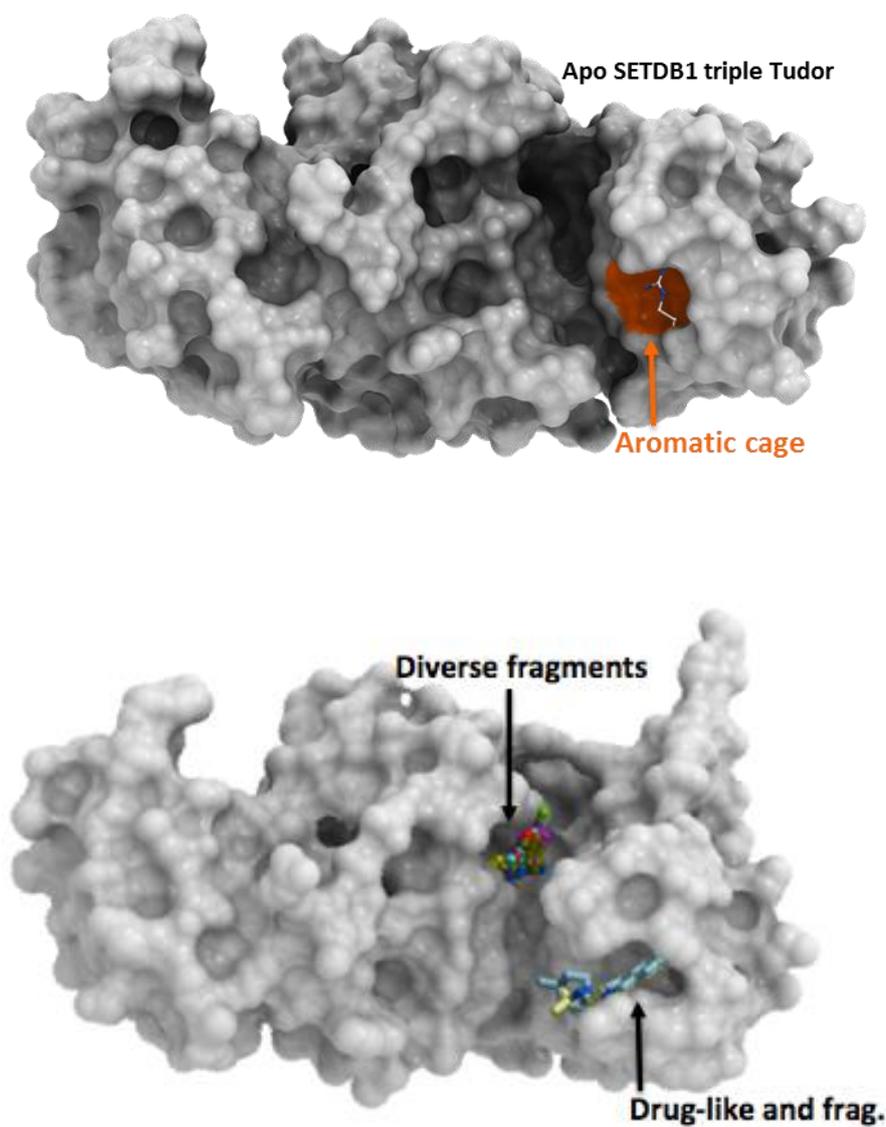
The SGC is a registered charity (number 1097737) that receives funds from AbbVie, Bayer Pharma AG, Boehringer Ingelheim, Canada Foundation for Innovation, Eshelman Institute for Innovation, Genome Canada through Ontario Genomics Institute [OGI-055], Innovative Medicines Initiative (EU/EFPIA) [ULTRA-DD grant no. 115766], Janssen, Merck KGaA, Darmstadt, Germany, MSD, Novartis Pharma AG, Ontario Ministry of Research, Innovation and Science (MRIS), Pfizer, São Paulo Research Foundation-FAPESP, Takeda, and Wellcome.

ADDITIONAL INFORMATION

Structure Files

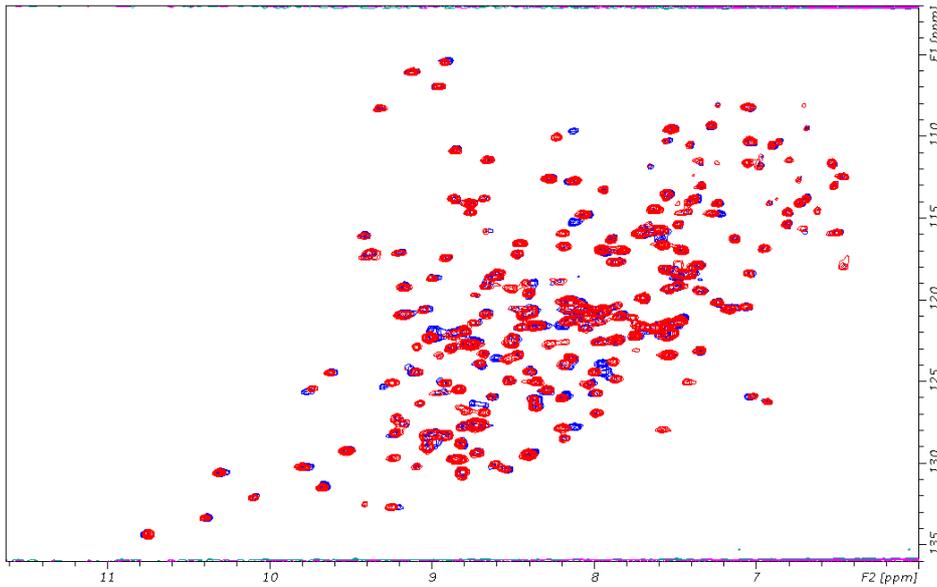
PDB ID	Structure Details
5KH6	Structures SETDB1 Tudor domain in complex with fragments
5KCO	Structures SETDB1 Tudor domain in complex with fragments
5KCH	Structures SETDB1 Tudor domain in complex with fragments
5KE2	Structures SETDB1 Tudor domain in complex with fragments
5KE3	Structures SETDB1 Tudor domain in complex with fragments

Materials and Methods

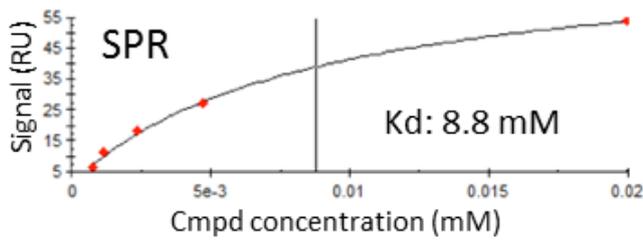


HSQC NMR of 10mM of MRT10241258a (blue) with SETDB1 and SETDB1 with DMSO (red)

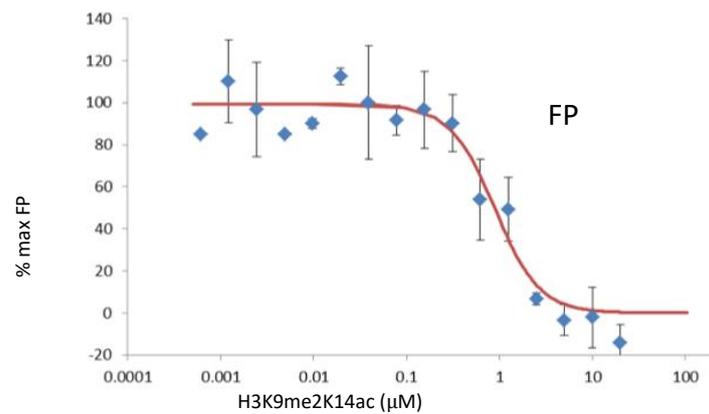
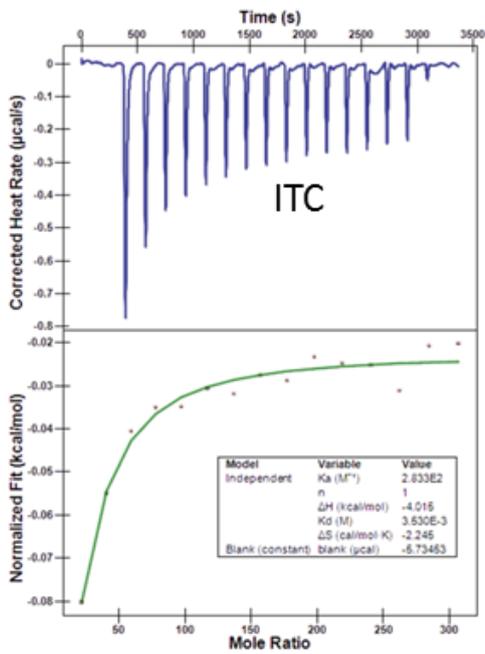
For more information regarding any aspect of TEPs and the TEP programme, please contact teps@thesgc.org



SPR data of one of the fragment (Chemical structure not presented)



ITC data of one of the fragment (Chemical structure not presented)



Experimental procedures

For more information regarding any aspect of TEPs and the TEP programme, please contact teps@thesgc.org

SPR Assay

Biotinylated SETDB1 captured onto a SA chip.

Buffer: 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20

5% DMSO Final

Compound stock concentrations varied from 400 μ M to 50 mM maintaining 5% DMSO final.

Flow rate: 25 μ L/min, compound contact time: 30 seconds at each concentration. Compounds were tested using Single Cycle Kinetics

ITC Assay

Buffer: 20 mM HEPES pH 7.5, 250 mM NaCl

Temperature 25C

Cell: SETDB1 0.08 mM

Syringe: Fragments 50-100mM

FP Assay

20 mM HEPES pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 0.01% TX100, 2 mM

DTT, containing 0.04 μ M of 5' FITC- H3K9me2K14ac (1-25) and 5 μ M SETDB1 at room temperature.

SETDB1 Tudor Protein Expression (used for crystallography, ITC, FP)

The expression construct for N-terminal His6-tagged SETDB1 Tudor domain (residues 197 – 403) subcloned into pET28a-MHL vector was transformed into BL21(DE3)-pRARE2 cells. The cultures were grown in Terrific Broth medium (TB) in the presence of 50 μ g/mL kanamycin and 34 μ g/mL chloramphenicol at 37 °C. When the OD₆₀₀ reached 1.5, the overexpression of SETDB1 Tudor was induced by addition of isopropyl-1-thio-D-galactopyranoside (IPTG), final concentration 0.5 mM, and incubated overnight at 16 °C. Next day, the cells were harvested by centrifugation at 12,227 \times g (10 min, 4C) and the cell pellets were flash frozen in liquid N₂ and stored at -80 °C.

¹⁵N SETDB1 Tudor Protein Expression (used for 1H-15N TROSY NMR)

The expression construct for N-terminal His6-tagged SETDB1 Tudor domain (residues 197 – 403) subcloned into pET28a-MHL vector was transformed into BL21(DE3)-pRARE2 cells. The cultures were grown in auto-induction M9 minimal medium containing 1.2 g/l 15N-ammonium chloride (Sigma 299251) as the sole nitrogen source using an auto-induction method described in (Adelinda A. Yee, Anthony Semesi, Maite Garcia, and Cheryl H. Arrowsmith (2014) Screening Proteins for NMR Suitability. Wayne F. Anderson (ed.), Structural Genomics and Drug Discovery: Methods and Protocols, Methods in Molecular Biology, vol. 1140, DOI 10.1007/978-1-4939-0354-2_13.). The medium contained 50 μ g/mL kanamycin and 34 μ g/mL chloramphenicol at the whole cultivation was done at 37 °C. The cells were harvested by centrifugation at 12,227 \times g (10 min, 4C) and the cell pellets were flash frozen in liquid N₂ and stored at -80 °C.

Biotinylated SETDB1 Tudor Protein Expression (used for SPR)

The expression construct was N-terminally biotinylated SETDB1 Tudor domain (residues 197 – 403) subcloned into pET28BIOH_LIC vector. Details of the expression and purification can be obtained from Ashley or Mani.

Purification of SETDB1 Tudor (same procedure for unlabeled and 15N-labeled, different MW)

The cell pellets were thawed and resuspended (1 g of cell pellet per 10 mL) in binding buffer (20 mM HEPES pH 7.5, 500 mM NaCl, 5 mM imidazole, 0.5 mM TCEP, 5% glycerol) with protease inhibitor (0.1 mM phenylmethyl sulfonyl fluoride, PMSF). The cell suspension was supplemented with 0.5% (w/v) CHAPS, 5 μ l of benzonase (EMD Millipore, cat. no. 70746), protease inhibitor cocktail (Roche) and the cells were sonicated on ice for 5 min total (10 s pulses with 5s interruptions). The lysate was clarified by centrifugation at 20,000 \times

g, 4 °C, 60 min and the resulting supernatant was filtered through 0.45 µm filter and applied onto 5 mL HisTrap HP column (GE). The column was washed with 10 CV of wash buffer (20 mM HEPES, pH 7.5, 500 mM NaCl, 40 mM imidazole, 0.5 mM TCEP, and 5% glycerol) and the protein was eluted using elution buffer (20 mM HEPES, pH 7.5, 500 mM NaCl, 250 mM imidazole, 0.5 mM TCEP, and 5% glycerol). Next the protein was applied on 26/60 Superdex 200 (GE) column equilibrated with 20 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM TCEP at flow rate 2 ml/min. As the final purification step, the protein was purified to homogeneity by ion-exchange chromatography on Source 30S column (10x10) (GE), equilibrated with 20 mM HEPES, pH 7.4, and eluted with linear gradient of NaCl up to 500 mM concentration (20CV). Purification yield was 1 mg of the protein per 1L of culture. The MW (26293.2 Da for unlabeled and 26 616.2 Da for the 15N labeled) of the purified construct was confirmed by LC/MSD TOF (Agilent).

BIOH-SETDB1 Expression (used for SPR experiments)

The expression construct was N-terminally biotinylated SETDB1 Tudor domain (residues 197 – 403) subcloned into pET28BIOH_LIC vector. Growth was done as usual but supplemented with 1 mg/ml of biotin.

Purification of BIOH-SETDB1 Expression (used for SPR experiments)

1. Preparation of Solutions

IMAC lysis buffer: 50mM Tris-HCl, 300 mM NaCl, 5% glycerol, 10 mM imidazole, 0.5 mM TCEP, 0.05% Triton X100 pH 8.0

IMAC wash 1 buffer: 50mM Tris-HCl, 300 mM NaCl, 5% glycerol, 10 mM imidazole, 0.5 mM TCEP, pH 8.0

IMAC wash 2 buffer: 50mM Tris-HCl, 300 mM NaCl, 5% glycerol, 20 mM imidazole, 0.5 mM TCEP, pH 8.0

IMAC elution buffer: 50mM Tris-HCl, 300 mM NaCl, 5% glycerol, 300 mM imidazole, 0.5 mM TCEP, pH 7.5

Gel filtration buffer: 20 mM HEPES, 300 mM NaCl, 5% glycerol, 0.5 mM TCEP, pH 7.5

2. Samples preparation

1). The frozen cell pellets are briefly thawed in warm water. Resuspend the cell pellets by adding Lysis buffer to final volume 100 mL/(per liter culture), Freshly add PMSF and Benzamidin to a working concentration of 1mM and add Benzonase to 5U/ml.

2). Sonication with program nr 1 (5s/7s, 10 min, ~100W) for lysate from 2 L cultivation.

3). The sonicated lysates are centrifuged for 60 min at 16000 rpm in the JLA 16.250 rotor. The soluble fraction is decanted and filtered through 0.45mm filters.

3. Purification

The 5 mL chelating columns and gel filtration columns were equilibrated with IMAC wash 1 buffer and Gel filtration buffer, respectively.

Purification programs:

Chelating 5ml GFS75 2nd wash 20CV level and slope

Chelating 5ml GFS200 2nd wash 20CV level and slope

1. Filtered samples are loaded to the IMAC columns on position 1-4 from sample inlets S1-S4

2. IMAC columns are washed by 10 CV of IMAC wash 1 buffer and 10 CV of IMAC wash 2 buffer.

3. IMAC columns are eluted by 5 CV IMAC elution buffer, and major peak is loaded to gel filtration columns on position 5.

4. The fractions from gel filtration column are checked by SDS-PAGE and pool the pure fractions together. The antigens are further validated by Mass spectrum.

5. The pure antigen samples are diluted or concentrated to 1 mg/ml and make 100 µL aliquots. The aliquots are flash freezed by liquid nitrogen and ship to binders lab with dry ice.

References

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