# IgM Purification

*(the fine print)*

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# *Increasing clinical interest in IgM*

*IgM monoclonal antibodies continue to attract increasing interest, especially in the field of cancer therapeutics.*

*This is due largely to their ability to easily discriminate glycosylation variants on key antigens that are either unrecognized or poorly recognized by IgG.*

# Increasing clinical interest in IgM

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Letter to the Editor

## IgM, not IgG, a key for HIV vaccine

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ABSTRACT

Although HIV infections induce a very strong humoral immune reaction with various neutralization activities and a well-defined cellular immunity, HIV vaccine development based on traditional approaches failed. The fact that neither specific antibodies nor activated $CD8^+$ cytotoxicity T-cells could provide primary protection in high risk populations raises the question concerning whether a specific vaccine is feasible. While the immune system as an intact defense system against HIV is ineffective or may even enhance the virus spread, a distinct small part of the system plays important role in delaying the progress of the disease. After carefully dissecting the different immune reactions against the virus, a new HIV vaccine strategy is indicated.

# DNA clearance: expectations

*Whatever the application, IgMs have several features that facilitate their purification.*

*Most bind strongly to anion exchangers (AX), cation exchangers (CX), hydrophobic interaction (HIC) media, and hydroxyapatite (HA).*

*These elements can be combined to yield a diversity of multi-step purification sequences, all of which should be expected to support extraordinary cumulative reduction of DNA.*

# DNA clearance: expectations

*DNA has the same charge as a cation exchanger. It should be repelled and appear exclusively in the flow-through during sample application. DNA reduction should be quantitative.*

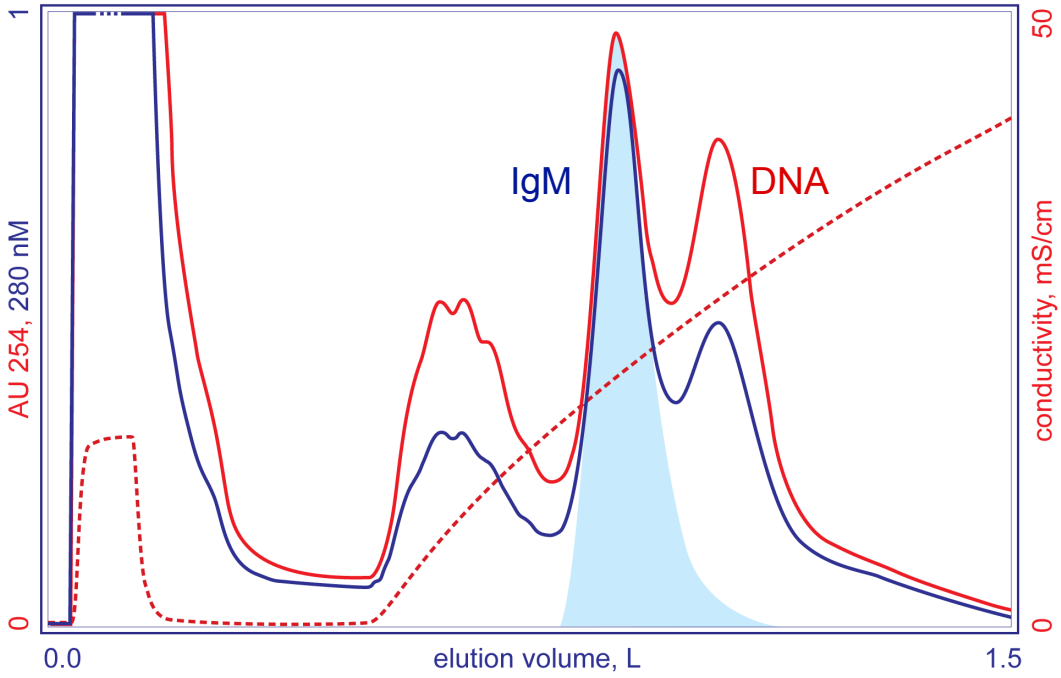
*DNA does not bind to weakly hydrophobic HIC media. IgM does. Reduction should be excellent.*

*DNA binds more strongly to AX than IgM. Reduction should be very good.*

*DNA binds more strongly to HA than IgM. Reduction should be good in phosphate gradients; excellent at high sodium chloride concentrations.*

# DNA clearance: the reality

## Capture on hydroxyapatite

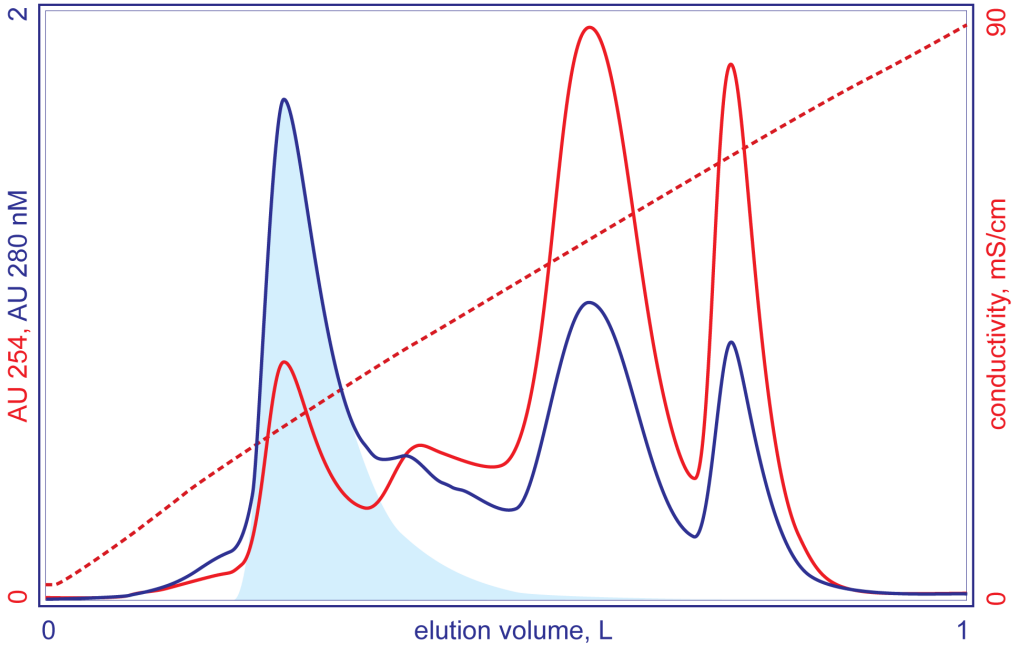



*CHT™ Type I, 40 µm*
*426 mL, XK 50*
*A: 10 mM $NaPO_4$ pH 7*
*B: 500 mM$PO_4$ pH 7*
*Elute: 20 CVLG A > B*
*The 254/280 ratio for pure IgM is about 0.5.*
*The ratio for pure DNA is about 2.0.*
*Even though the bulk of the DNA elutes after IgM, the IgM peak co-elutes with a significant DNA load.*

*The absorbance differential between equal concentrations of IgM at 280 nm and DNA at 254 nm is about 5000x, so when absorbance at 280 equals absorbance at 254, as roughly at the IgM peak, the DNA/IgM ratio would be about 200 ng/mg (200 ppm).*

# DNA clearance: the reality

## Anion exchange of hydroxyapatite eluate




*Fractogel® TMAE HiCap*
*118 mL, XK 50*
*Load: 1 L HA eluate*
*Estimated purity ~90%*
*A: 25 mM Tris, pH 8*
*B: A + 1 M NaCl*
*Elute: 10 CVLG A > B*
*Estimated proportion of IgM shown in blue.*

*If feed stream purity is really 90%, why the heterogeneity?*

*The leading shoulder contains non-IgM protein contaminants left over from the HA capture step. The 254/280 ratio of the first peak is close to the value for purified IgM. Later peaks exhibit an increasing 254/280 ratio, suggesting co-elution with an increasing proportion of DNA.*

# DNA complexation

*The AX profile demonstrates that DNA is not merely co-eluting with IgM from the HA step; it is complexed.*

*Why? How?*

*DNA is itself a high density weak cation exchanger, with two phosphate-bound negatively charged oxygen atoms on each side of the molecule at every base-pair node.*

*IgMs frequently bind CX so strongly that they support high capacity capture even under physiological conditions, so it is inevitable that they should aggressively bind DNA as well.*

*But then how do the complexes survive high conductivity at high pH?*

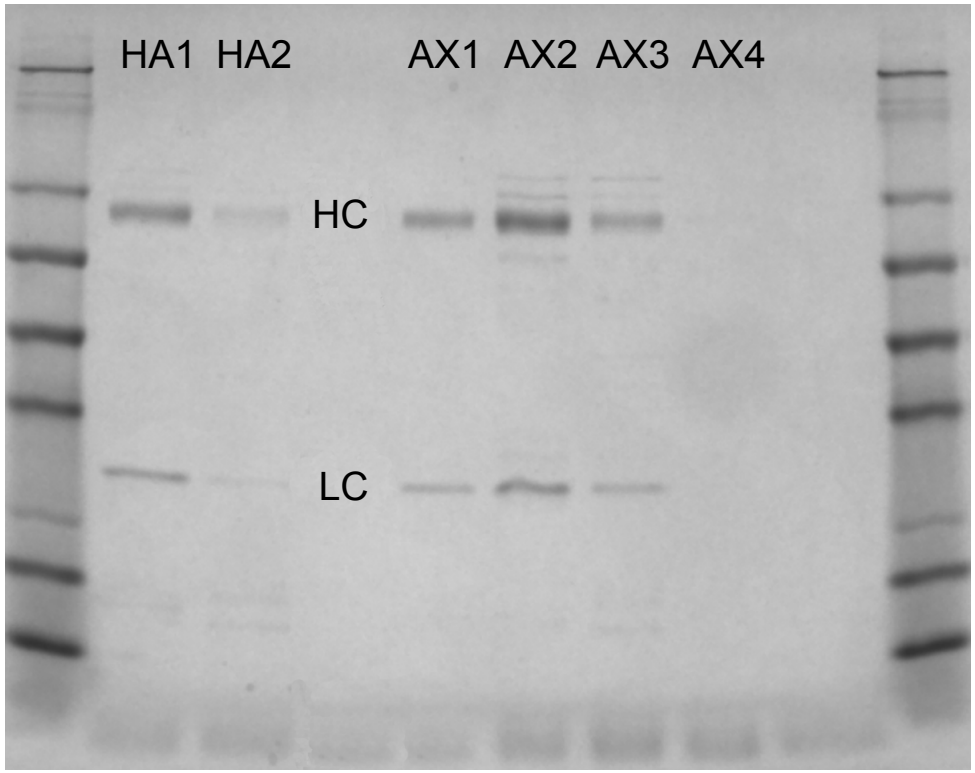
# DNA complexation

*DNA phosphoryl oxygen atoms should be able to form hydrogen bonds with hydrogen residues on the peptide backbone (and elsewhere) on IgM.*

*Given the combined potential of strong electrostatic interactions and hydrogen bonding, it is not surprising that IgM:DNA complexes easily survive the range of conditions traditionally employed in protein purification.*

# DNA complexation

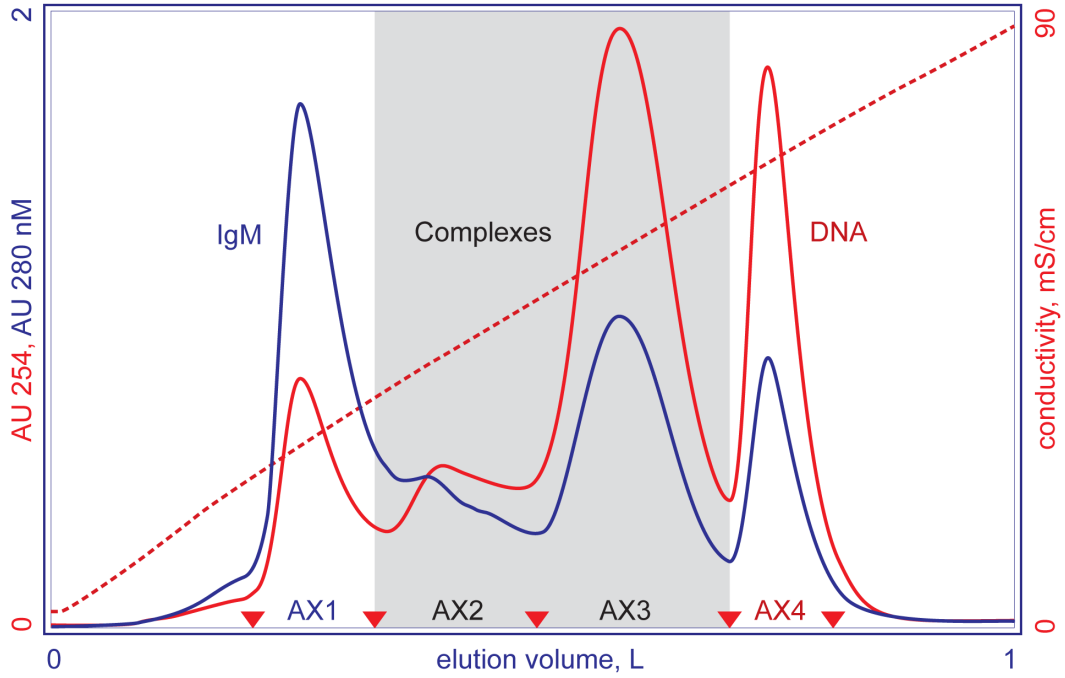
## *Reduced SDS-PAGE of selected HA and AX fractions*




*5-15% gradient gel Coomassie brilliant blue. HA1 is the "IgM" peak. HA2 is the "DNA" peak. Note the contaminating protein bands in the later AX fractions. This cautions that complexes include more than just IgM and DNA. Histones are likely constituents but any solute able to bind nonspecifically to IgM or DNA is a potential candidate. This elevates the priority for complex-dissociation or removal.*

# DNA complexation

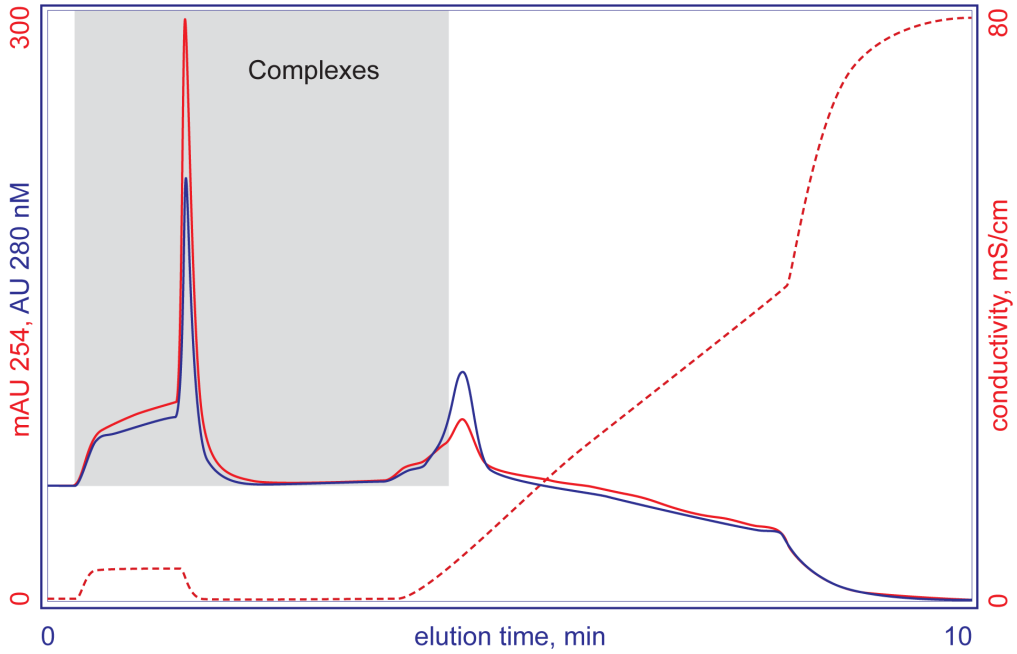
## Tentative identification of AX peaks




*PAGE results suggest the likely composition of the peaks eluting from TMAE. The first peak is nearly "pure" IgM, and the last nearly "pure" DNA. The remainder, left to right, appear to represent a progression in the relative proportion of DNA, possibly correlating with the size of DNA in each fraction.*

# DNA complexation

## How does complexation affect CX?



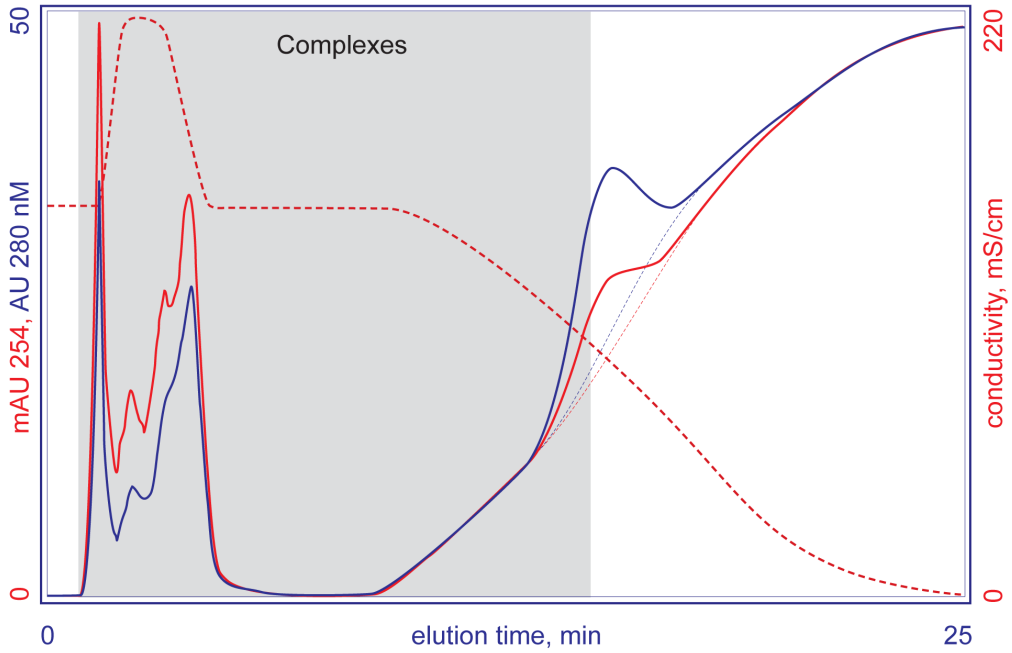

*CIM $SO_3$, 4 mL/min*
*A: 20 mM Hepes, pH 7*
*B: A + 1 M NaCl*
*EQ: 100% A*
*Load: TMAE complexes*
*Wash: 100% A*
*Elute: 60 CVLG to 50%B*
*Clean: 100% B*

*All of the flow-through and the leading side of the elution peak are complexed.*

*The flow-through spike at the end of the sample load coincides with a decrease in conductivity. This suggests that the spike is caused by increased charge repulsion between exchanger $SO_3$ groups and negatively charged phosphates on DNA fragments complexed to IgM. Product recovery is obviously poor.*

# DNA complexation

## How does complexation affect HIC?



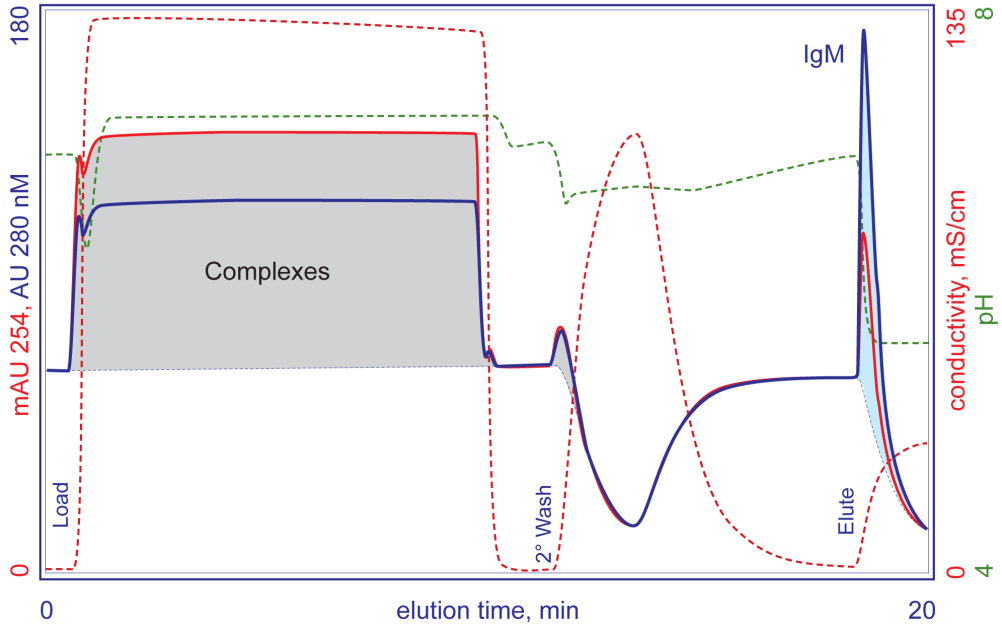

*Toso PPG HW75*
*Post-TMAE complexes:*
*>95% IgM in 3.2 M NaCl*
*Equilibrate in 50 mM Hepes, 1 M ammonium sulfate, pH 7*
*Elute 10 CVLG to 50 mM Hepes, pH 7*

*Flow-through fractions are DNA dominant. Binding fractions appear to be IgM dominant.*

*Despite the sample having been applied in 3.2 M NaCl, substantial UV absorbance was unretained. The 254/280 ratio of the leading shoulder on the elution peak suggests the presence of complexes. DNA apparently reduces net hydrophobicity of complexes, which is consistent with the inability of DNA to bind weakly hydrophobic media like PPG.*

# DNA complexation

## How does complexation affect affinity chromatography?

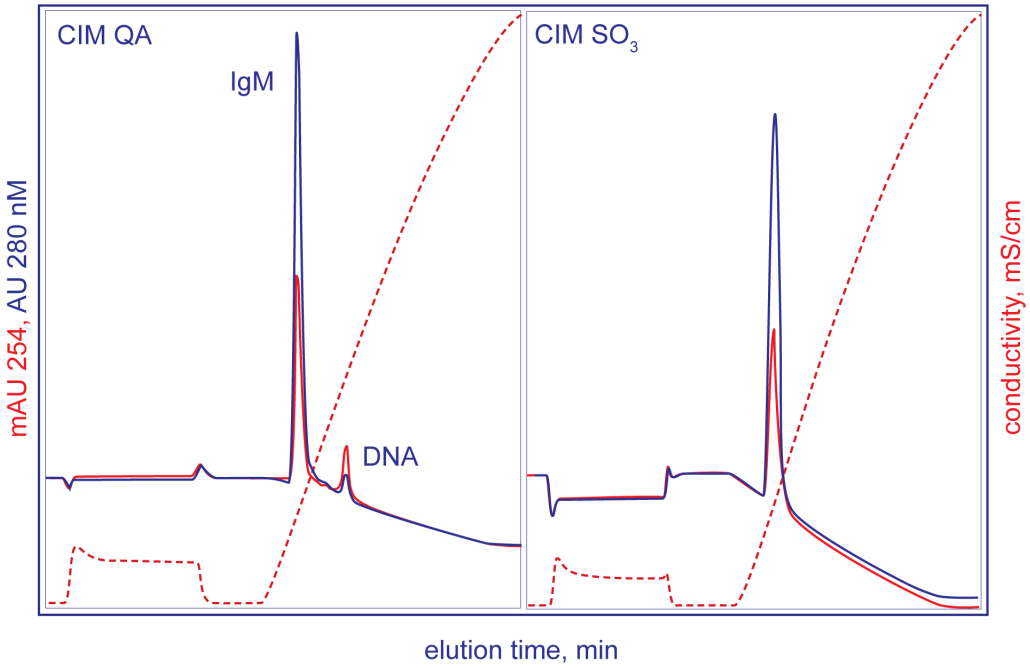



*CaptureSelect® anti-lambda light chain.*
*EQ: 50 mM Hepes, 100 mM NaCl, pH 7 (HBS)*
*Load: TMAE complexes, NaCl added to ~1.5 M*
*Wash1: HBS*
*Wash2: 2M urea, 1.5 M NaCl, 10 mM EDTA, pH 7*
*Wash3: HBS*
*Elute: 500 mM arginine, 100 mM MES, pH 5.5*

*Complexed DNA apparently blocks immuno-recognition by the affinity ligand — which means it probably also has consequences for IgM quantitation by ELISA. As with HIC, elevated salt concentration is unable to substantially dissociate complexes. Even if it did, the economics of large volume salt addition might not be favorable.*

# DNA complexation

## How does affinity chromatography affect complexation?

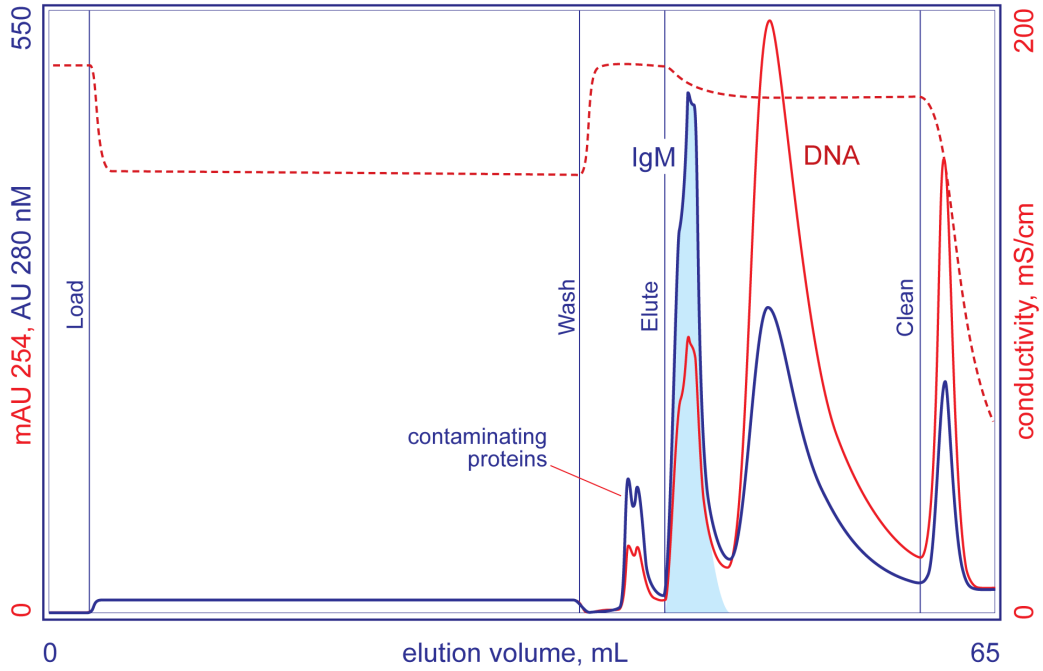



*CIM QA & CIM $SO_3$*
*EQ: 50 mM Hepes, pH 7*
*Load: affinity eluate*
*Wash: EQ buffer*
*Elute: LG to A + 2 M NaCl*
*These profiles highlight one of the conveniences of IgMs: they often bind so well to both types of exchangers that you can initially apply the same buffers and separation conditions on both. Optimize later.*

*Affinity with a strong secondary wash does not totally remove complexed DNA but should reduce it to a consistent level despite feed stream variations, and put it into a range where it can be managed effectively by other methods. Note that residual DNA revealed by AX co-elutes invisibly from CX, making AX the better tool for process characterization.*

# DNA complexation

*Can competitive dissociation be enhanced in a high salt environment?*



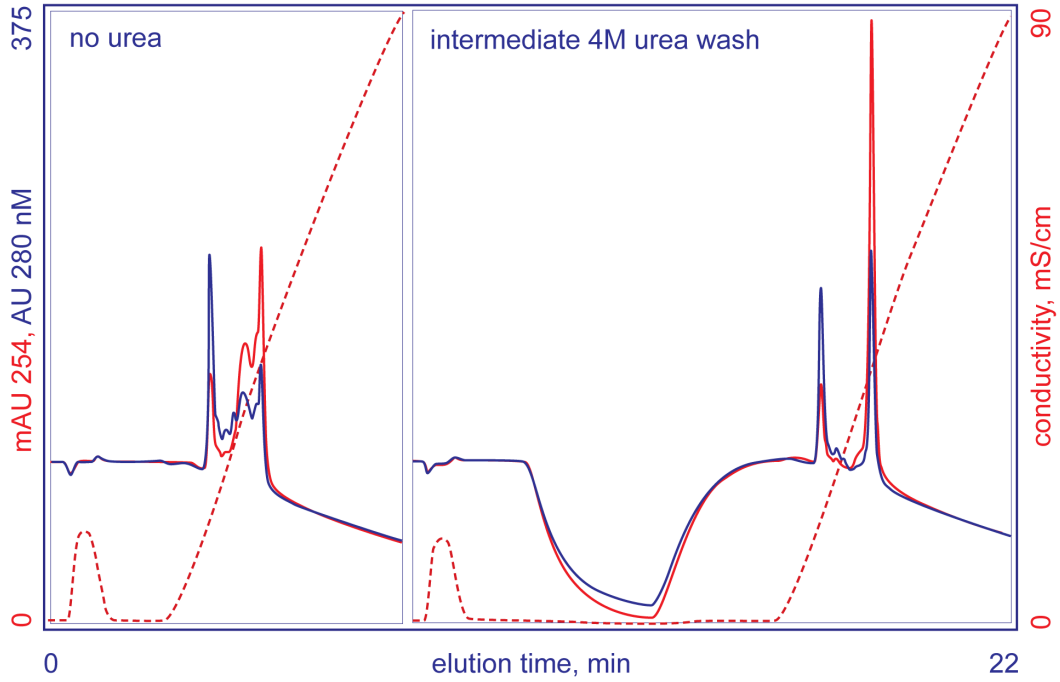

*HA, CHT Type II 40 micron*
*EQ: 2 M NaCl, 10 mM $NaPO_4$ pH 7*
*Load: flow-through from affinity application (~1.5 M NaCl)*
*Wash: EQ buffer*
*Elute: 2 M NaCl, 250 mM $NaPO_4$ pH 7*
*Clean: 500 mM $NaPO_4$ pH 7*

*DNA binds HA by coordination of its phosphoryl residues with HA calcium. This mechanism is unaffected by NaCl.*

*HA in the presence of 2 M NaCl achieves near-complete dissociation. This lends support to the notion that competitive dissociation —in this case via DNA's affinity for calcium— is crucial for effective decomplexation. NaCl facilitates dissociation by suppressing the electrostatic attraction between DNA and IgM. Compare with the HA profile on slide 6.*

# DNA complexation

*Can competitive dissociation be enhanced in a low salt environment?*

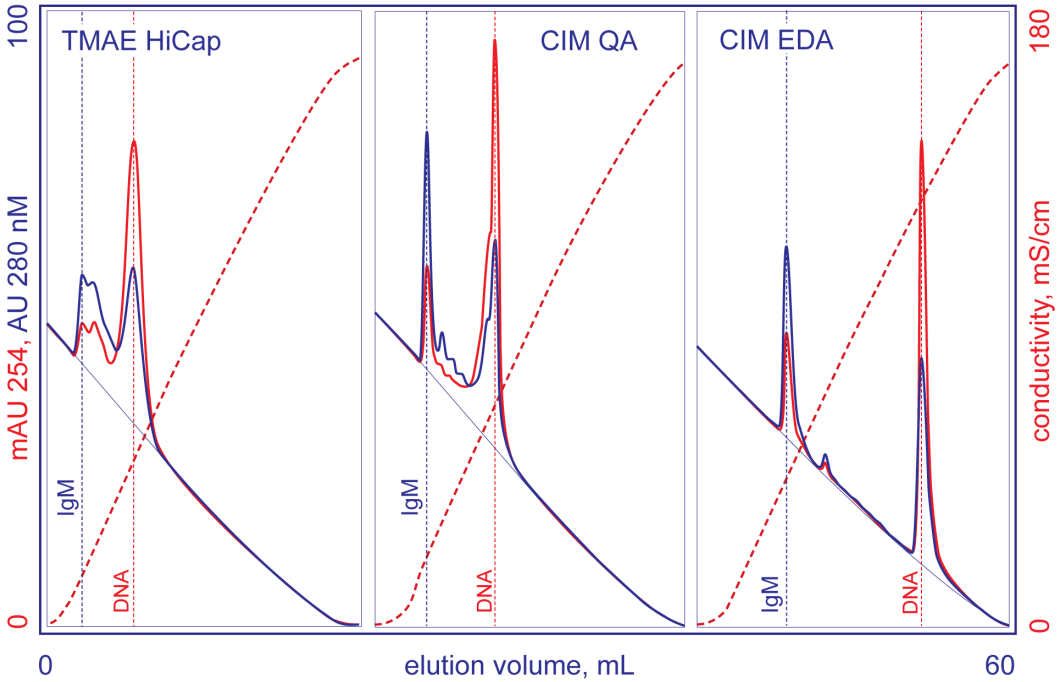



*CIM QA, 4 mL/min*
*Buffer A: 20 mM Tris, pH 8*
*Buffer B: A + 1 M NaCl*
*EQ: buffer A*
*Load: TMAE complexes*
*Wash1: buffer A*
*Wash2: A + 4 M urea*
*Wash3: buffer A*
*Elute: 60 CVLG to buffer B*
*Urea is a nonionic hydrogen donor/acceptor. As such it can suspend hydrogen bonds but not interfere with electrostatic interactions.*

*Loss of hydrogen bonds weakens the association between IgM and DNA. In this experiment, the dissociative effect of urea occurred exclusively at low conductivity, ~2 mS/cm. This conserves the full capability of electrostatic interactions to competitively dissociate IgM/DNA complexes. Urea also works at higher conductivity, but electrostatic dissociation is impaired.*

# DNA complexation

## Do all AX media support equivalent decomplexation?




*TMAE HC, 1 mL, 5x50 mm*
*CIM QA & CIM EDA, 1 mL*
*All at 1 mL/min*
*A: 50 mM Hepes, pH 7*
*B: A + 2 M NaCl*
*EQ: 100% A*
*Load: TMAE complexes*
*Wash: 100% A*
*Elute: 20 CVLG to 100%B*

| EL/ms | IgM | DNA | Delta |
|---|---|---|---|
| TMAE | 23.7 | 54.8 | 31.0 |
| QA | 25.8 | 67.0 | 41.2 |
| EDA | 43.3 | 117.7 | 74.8 |

*TMAE is barely able to dissociate any IgM from complexes. QA recovers the majority, but a spectrum of complexes remain. EDA dissociates complexes almost completely, plus the separation is more than double that achieved by TMAE.*

# DNA complexation

*Why do monoliths support more effective decomplexation than porous particle exchangers?*

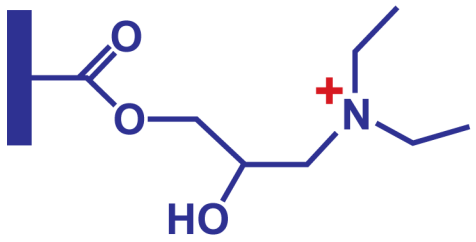
*1. DNA binding capacity is 10-40 times higher on monoliths than on porous particles. Thus DNA sufficient to saturate a particle-based anion exchanger would leave 90–97.5% of a monolith's capacity available to dissociate complexes.*

*2. Monoliths have higher ligand density than most porous particle exchangers. This may enhance their ability to competitively dissociate DNA:protein complexes.*

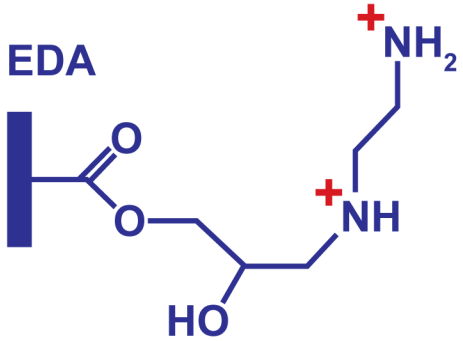
# DNA complexation

*Why does EDA decomplex more effectively than QA?*

QA




EDA

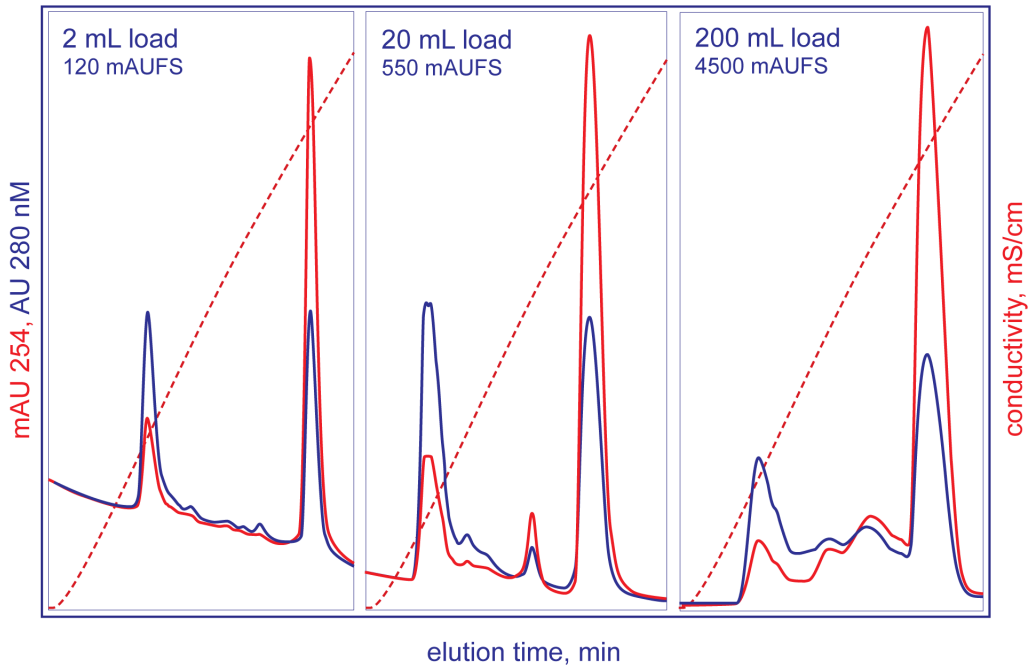



*QA bears a single strong anion exchange group. EDA bears two weak anion exchange groups. Assuming equivalent ligand density, this effectively doubles the charge density.*

*Note that QA bears 15 hydrogen residues potentially capable of participating in hydrogen bonds; EDA 12, although in both cases the number of hydrogens sterically accessible to a charge-bound DNA or protein molecule may be lower.*

# DNA complexation

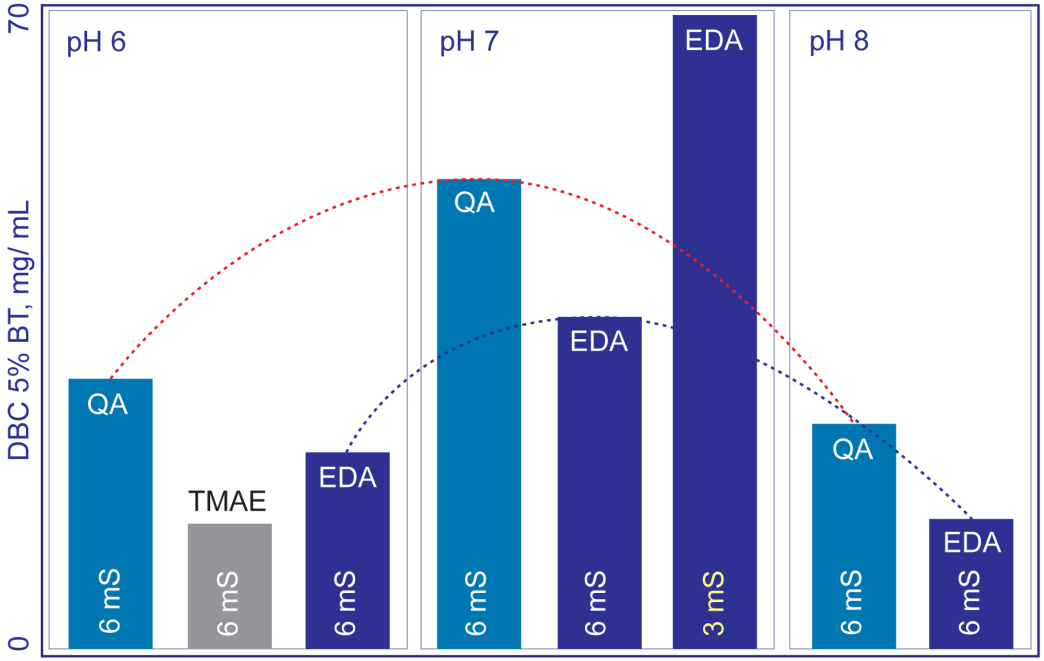
## Does complexation influence capacity?




*CIM EDA, 4 mL/min*
*A: 50 mM Hepes, pH 7*
*B: A + 2 M NaCl*
*EQ: 100% A*
*Load: TMAE complexes, volume as indicated*
*Wash: 100% A*
*Elute: 10 min LG to 100%B*

*These results show that decomplexation requires excess capacity, which means that complexation reduces usable capacity. It also reduces recovery of decomplexed IgM. To optimize both, reduce complex levels as much as possible in previous steps.*

# DBC of decomplexed IgM




*All samples were loaded by in-line dilution, with an over-buffered diluent to achieve the target pH. For example, the pH 6 set was initially buffered with 20 mM Hepes, pH 7, and diluted through the chromatograph with 100 mM MES, pH 6. Final conductivity was determined by the in-line dilution factor. Flow rate for monolith experiments was 4 mL/min; for TMAE, 1 mL/min.*

*The similar pH trends for CIM QA and CIM EDA at 6 mS suggest that a factor other than the charge characteristics of the protein and exchangers governs maximum capacity. This factor may be IgM solubility. The low value for TMAE supports this notion, since pre-column sample residence time in the target buffer was 4 times longer than with the monoliths. This would favor precipitation, encumbering pore accessibility. Lower conductivity (3 mS) further favors precipitation, but the speed of monoliths (EDA) remains ahead of the solubility curve.*

# *Is complexation a broader issue?*

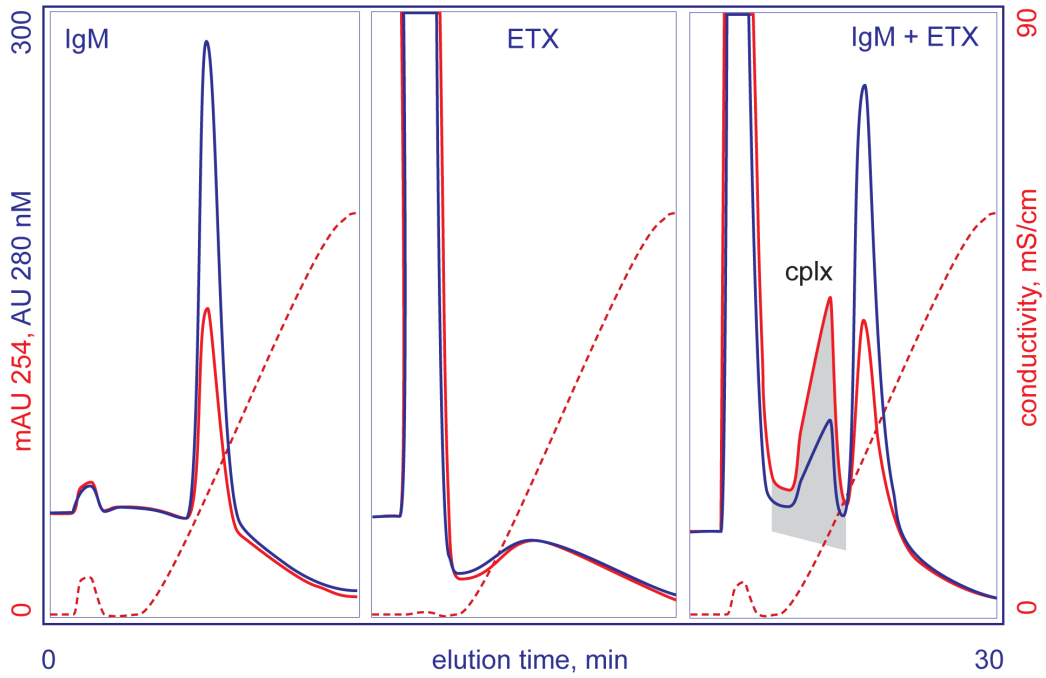
*DNA complexation with IgG (picogreen)*

| ***Process step*** | ***ppm DNA*** |
|---|---|
| *PA eluate* | *32* |
| *CX eluate* | *33* |
| *AX eluate* | *1* |

*PA: protein A. Note that as with IgM, CX is ineffective for DNA complex removal from IgG, while AX reduces it to the detection limit of the assay. IgGs with high isoelectric points have been observed to carry 190 ppm DNA across a CX capture step. If this can happen with IgG, it can certainly occur with other products. Data from http://www.validated.com/revalbio/pdffiles/Biobetter.pdf. See also Luhrs et al, J. Chromatogr. B, 877 (2009) 1543–1552*

# Is complexation a broader issue?

## IgM complexation with endotoxin




*CIM SO3, 4 mL/min*
*A: 20 mM Hepes, pH 7*
*B: A + 1 M NaCl*
*EQ: 100% A*
*Load: as indicated*
*Wash: 100% A*
*Elute: 60 CVLG to 60%B*
*ETX from S. typhimurium*

*Like DNA, endotoxins strongly absorb UV; 254 more than 280 nm.*

*Endotoxins are heavily carboxylated and lightly aminated in addition to being phosphorylated. The lipid A portion is also extremely hydrophobic, altogether endowing endotoxins with a wide range of mechanisms by which they can form persistent complexes with other biomolecules. If a product binds DNA and ETX, assume it also binds virus and other contaminants. Assume equally that DNA plasmids and viral products complex with an assortment of contaminants.*

# *Economic and regulatory concerns*

*Complexation depresses the ability of every purification method to reduce DNA levels. Accordingly, it compromises the ability of the overall process to achieve clinically acceptable DNA levels.*

*Complexes compete for binding substrate, thereby reducing product binding capacity, requiring larger columns, more buffer, and more time for each process step.*

*Complexes removed during the process — for example as flow-through during affinity, CX or HIC, or discarded eluate during any method — contribute directly to product loss, the most expensive of all process deficiencies.*

# *Economic and regulatory concerns*

*DNA levels in cell culture supernatants vary, depending on the proportion of dead cells at termination and lysis of living cells during harvest.*

*The level of DNA is likely a primary determinant of the level of complexation going into the purification process.*

*If a purification process lacks excess capacity to remove DNA, then DNA levels in the initial feed stream constitute an uncontrolled process variable, with the potential to compromise reproducibility of product quality.*

# *Recommendations*

*Whenever approaching a new purification development process for **any** product, assume that complexation is an issue.*

*With DNA complexation in particular, be especially wary with products that bind strongly to cation exchangers.*

*Know the symptoms:*

- *Unexpected product flow-through with CX, HIC, or affinity.*
- *Unexpected product in the high-salt cleaning step from AX.*
- *254/280 ratios greater than decomplexed product.*
- *The presence of contaminants that don't make sense based on their properties and the fractionation method.*

# Recommendations

*If complexation is confirmed as an issue, recognize that managing it may become the dominant consideration in the overall design and development of the purification process.*

*Be prepared to apply dissociative tactics at every point that will tolerate them…*

*…keeping in mind that if you have an issue with DNA, you may have an issue with endotoxin and other contaminants as well.*

# Recommendations

*Prioritize application of purification methods with strong complex-dissociative ability: AX in the presence of urea, HA in the presence of NaCl (and/or urea), affinity with enhanced washing steps.*

*Put the strongest decomplexing methods first to maximize product recovery, and to enhance the ability of subsequent steps to consistently achieve target DNA levels.*

*Restrict dissociative agents to a pre-elution wash if you can; in the elution only if necessary; and avoid them in the feed stream since they will probably reduce binding capacity.*

# Endnotes

*Your greatest enemy will hide in the last place you would ever look.*

*—Gaius Julius Caesar*

*Thanks to BAC BV for providing CaptureSelect anti-lambda light chain affinity chromatography media, to EMD Chemicals for TMAE HiCap, and to Tosoh Bioscience Inc. for providing experimental wide-pore PPG HIC media. Special thanks to George Rodriquez at Avid BioServices for running PAGE gels.*

*Copies of this presentation can be downloaded at www.validated.com*