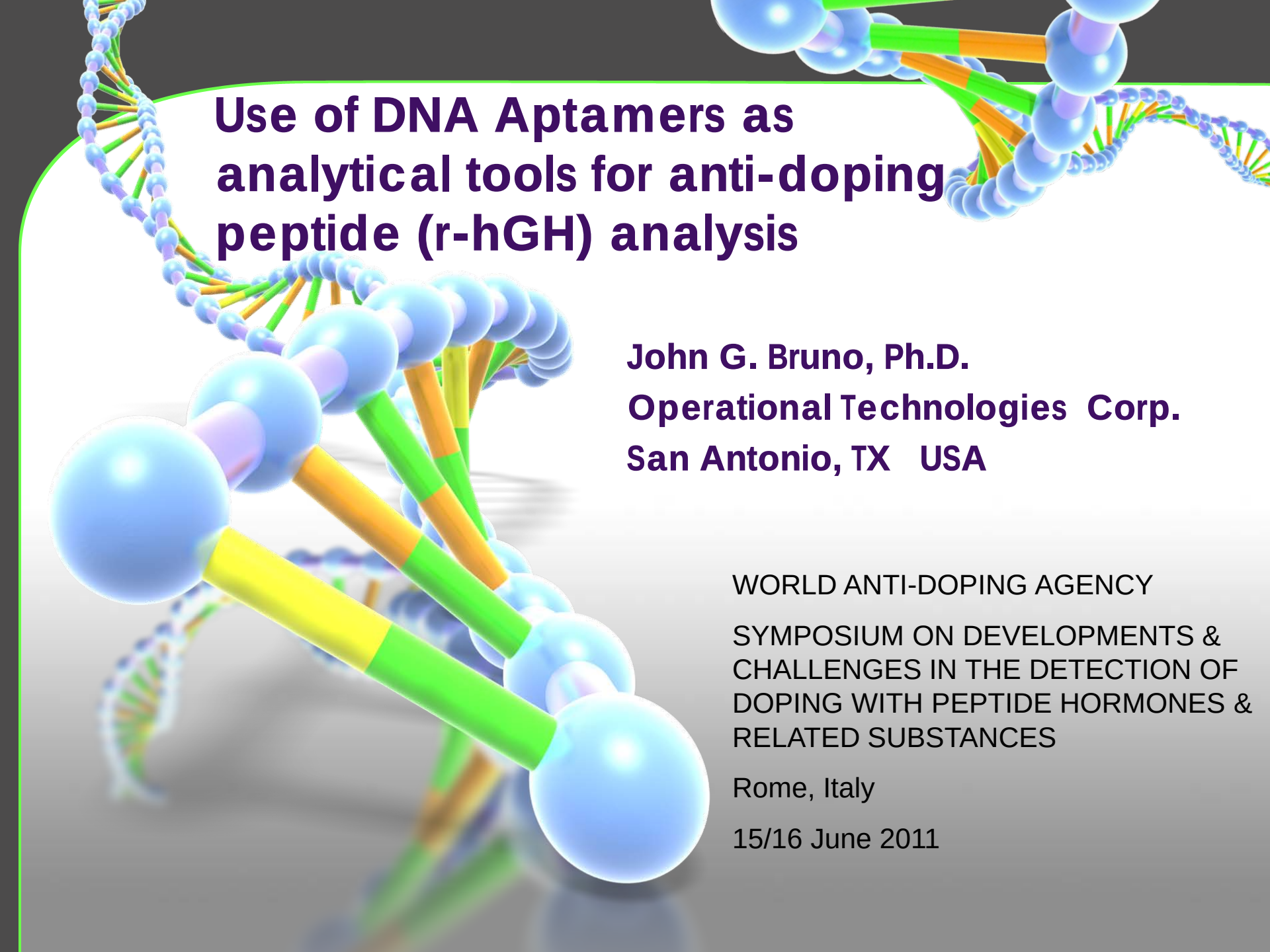


Use of DNA Aptamers as analytical tools for anti-doping peptide (r-hGH) analysis

John G. Bruno, Ph.D.
Operational Technologies Corp.
San Antonio, TX USA

WORLD ANTI-DOPING AGENCY

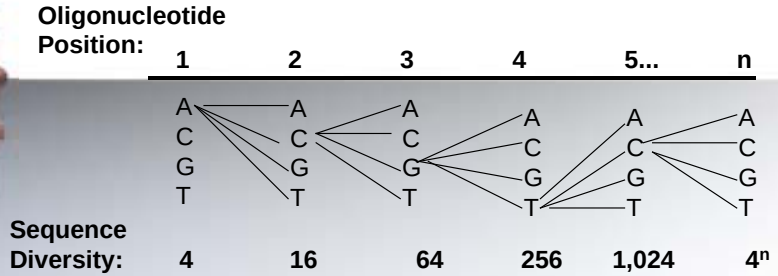
SYMPOSIUM ON DEVELOPMENTS & CHALLENGES IN THE DETECTION OF DOPING WITH PEPTIDE HORMONES & RELATED SUBSTANCES

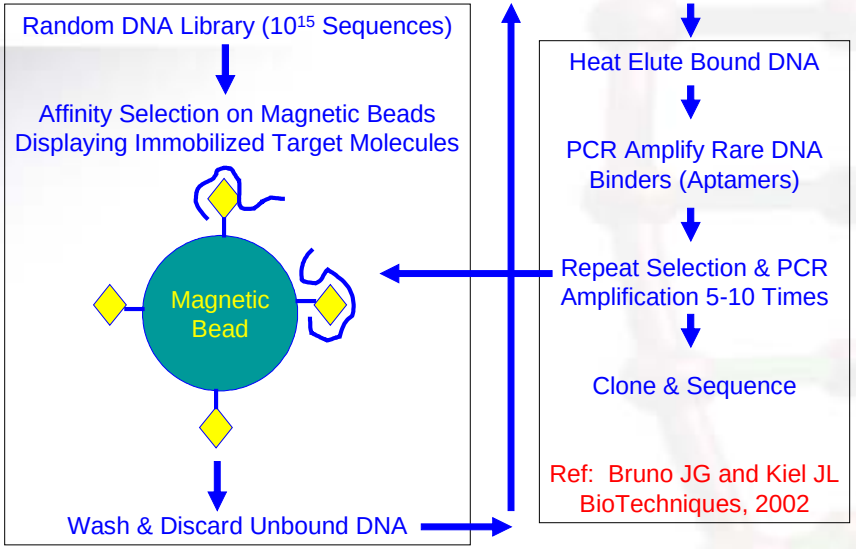
Rome, Italy

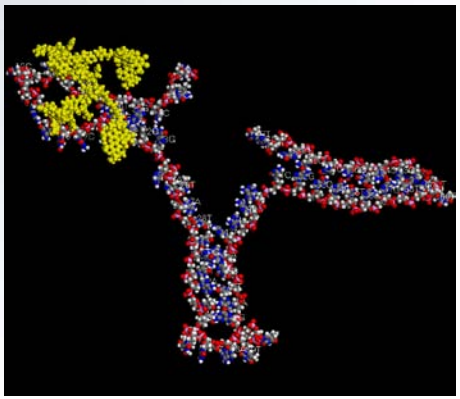
15/16 June 2011

Aptamers

- DNA/RNA molecules that mimic antibodies
- Made entirely in vitro, obviate host animals
- Can produce higher affinity & specificity
- Pioneered by Gold & Ellington, early 1990's

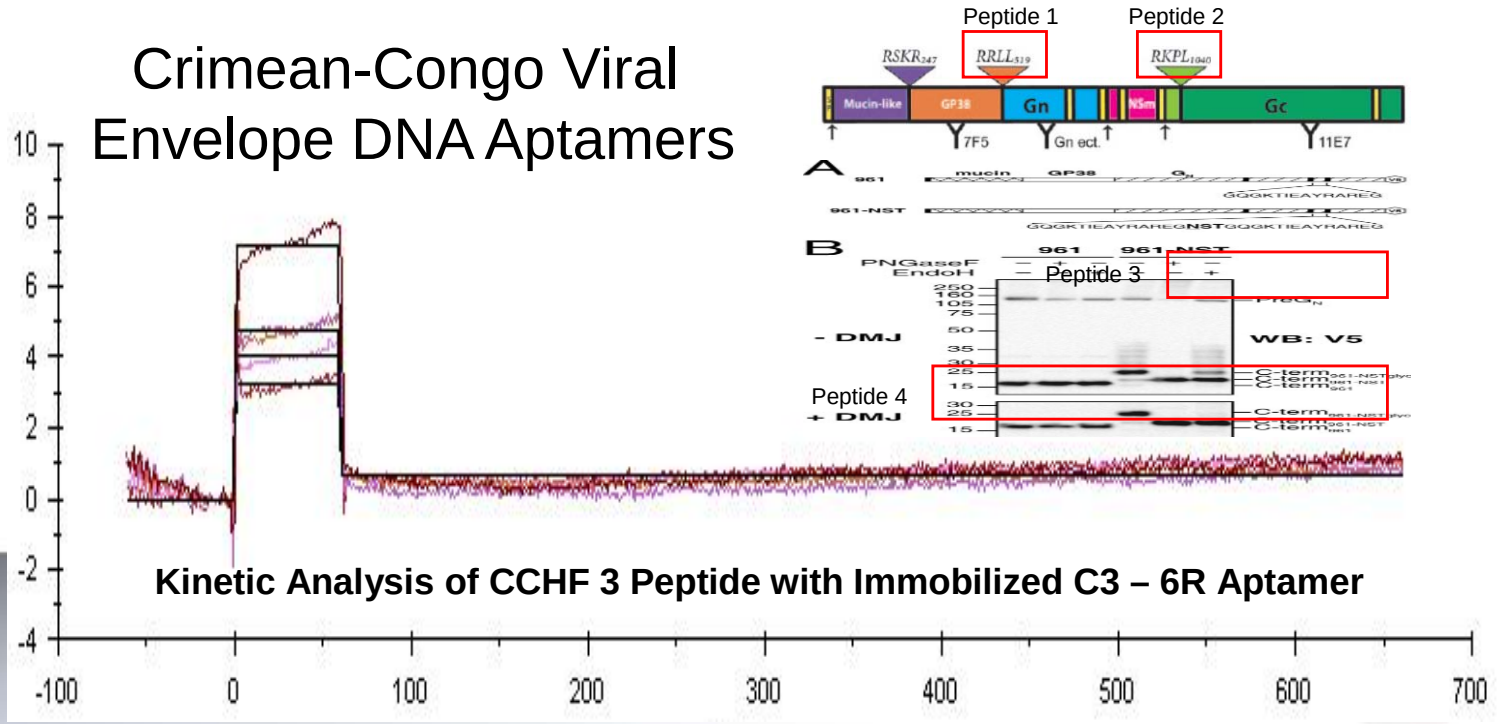






Potentially Higher Affinity vs. Antibodies

Crimean-Congo Viral Envelope DNA Aptamers



k_a (1/Ms) = 2.65 x 10^5

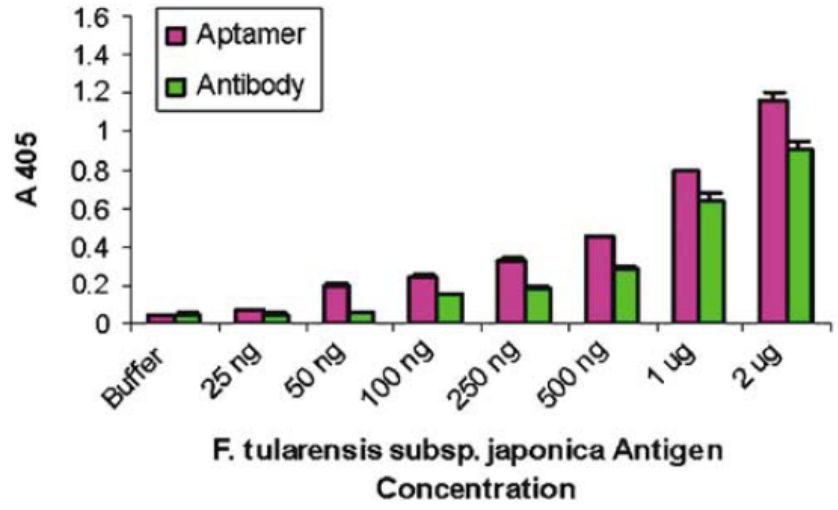
k_d (1/s) = 6.27 x 10^{-7}

K_A (M^{-1}) = 4.22 x 10^{11}

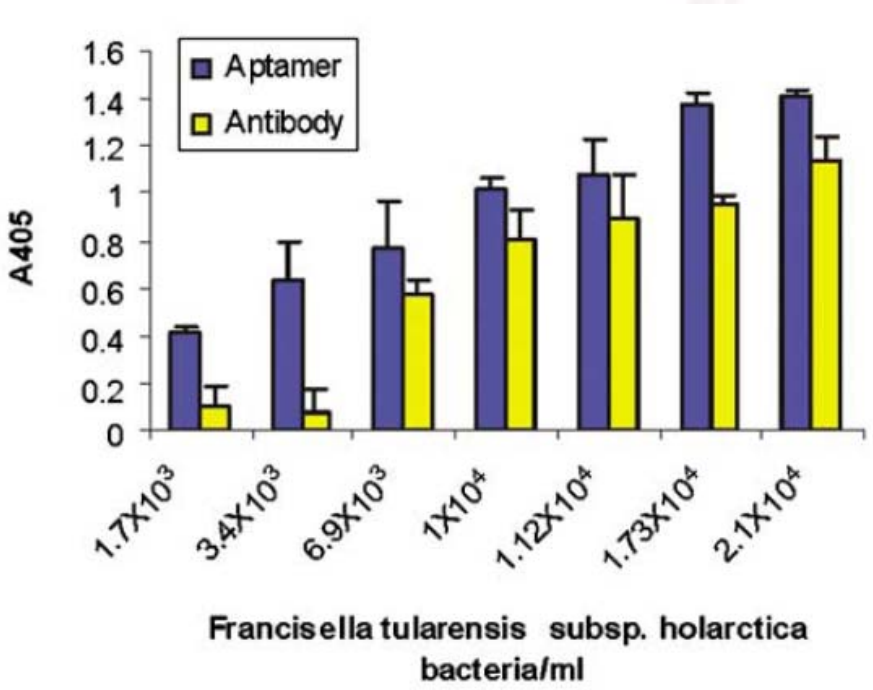
K_D (M) = 2.37 x 10^{-12}

Side-by-Side Comparison w/ Antibody

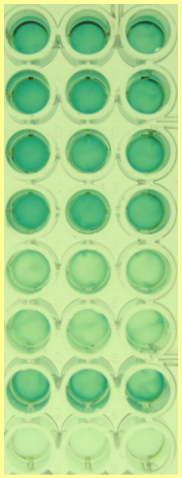
DNA aptamers for *F. tularensis* antigen
J Vivekananda and JL Kiel

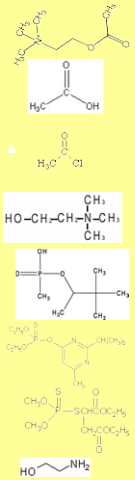


Vivekananda & Kiel
Lab Invest. 2006



Aptamer specificity



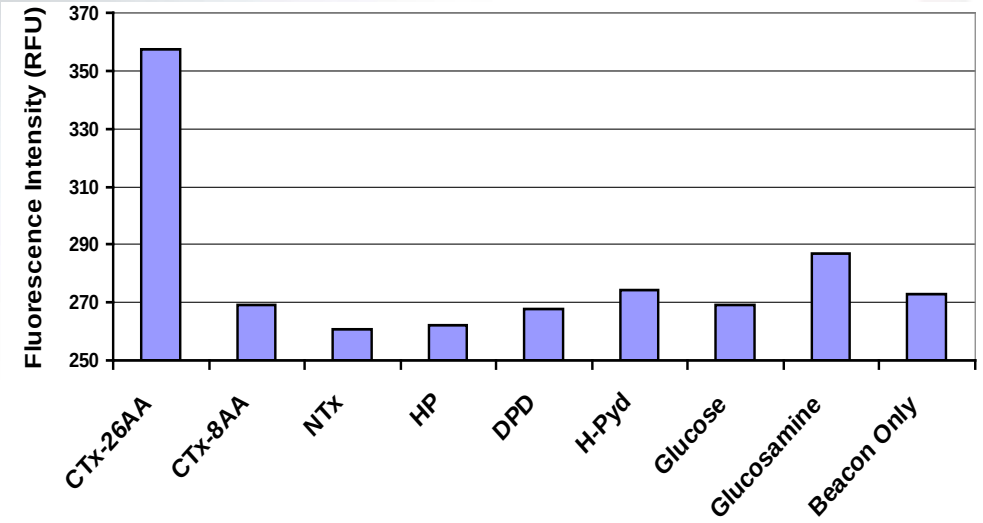


- Aptamers were selected against ACh immobilized in middle of molecule
- Aptamers bind acetyl & choline moieties
- Aptamers cross-react with other ester moieties
- Aptamers did not cross-react with unrelated small molecules such as Pinacolyl-MPA (GD acid) Or Diazinon

Acetylcholine Aptamers

Bruno J.G., et al. *In Vitro Cell & Develop Biol –Animal* 2008

C-telopeptide from Bone Collagen (CTx) Aptamers for NASA



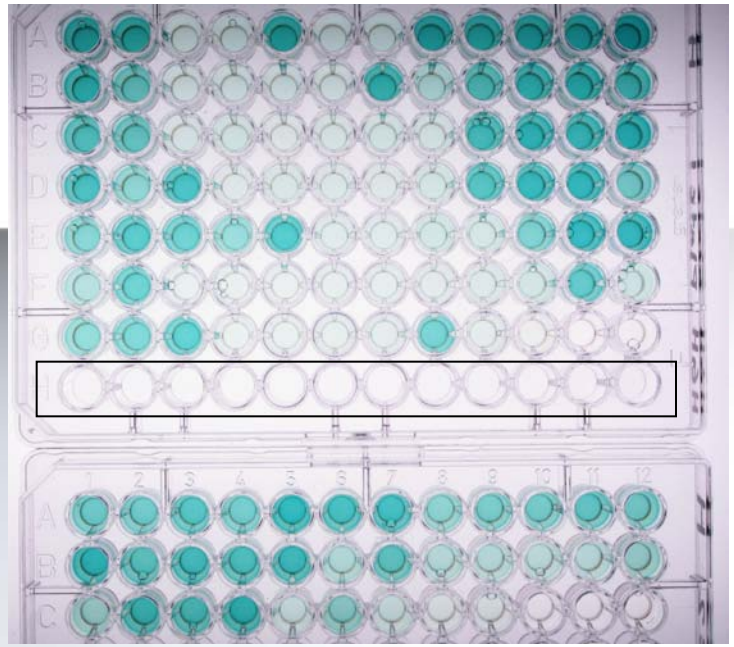
Rationale for WADA Pilot Study

- Hepner et al., had shown modifications (deamidations, amino acid substitutions, etc.) in about 2% of Genotropin r-hGH by mass spectral analysis
- Research grade r-hGH from Genway Biotech (San Diego, CA) had N-terminal methionine (signal sequence) to distinguish it (proof-of-concept)
- Aptamers might be found that bind tighter to these modified regions of r-hGH and discriminate r-hGH from natural hGH
- Used a negative selection or “adsorption” approach (anti-r-hGH aptamer pool exposed to natural hGH)
- These could be exploited in various assay formats from serum and urine samples for ultrasensitive (sub-picogram) r-hGH detection

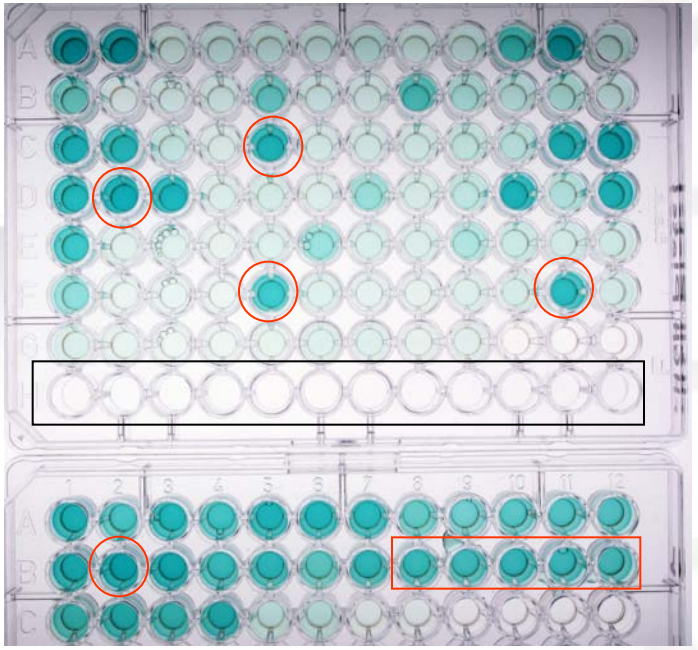
Consistent Differences in ELASAs Between hGH and r-hGH Aptamers

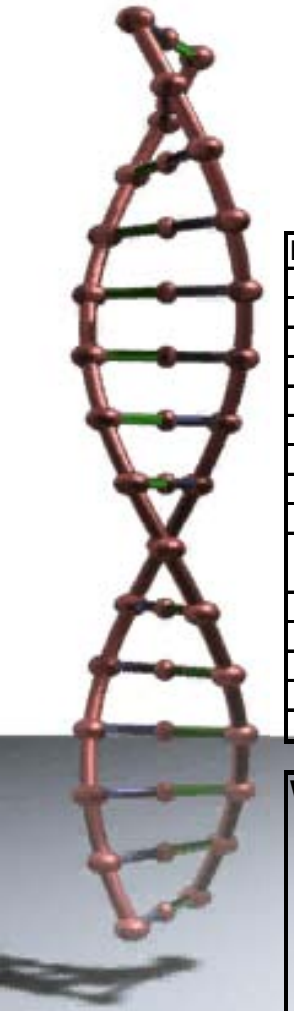
DNA Aptamers

vs. 1 µg Natural hGH



vs. 1 µg Recombinant-hGH





Tabulated Mean Δ Abs 405nm ≥ 0.5 (N = 3)

ELASA Plate Scheme / Layout

PLATE 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	rH-31F	rH-31R	rH-34F	rH-34R	rH-35Fa	rH-35Ra	rH-35Fb	rH-35Rb	rH-36Fa	rH-36Ra	rH-36Fb	rH-36Rb
B	rH-37F	rH-37R	rH-38F	rH-38R	rH-39F	rH-39R	rH-45/60F	rH-45/60R	rH-48F	rH-48R	rH-49F	rH-49R
C	rH-50F	rH-50R	rH-52F	rH-52R	rH-54F	rH-54R	rH-55F	rH-55R	rH-57F	rH-57R	rH-58F	rH-58R
D	Ar-31F	Ar-31R	Ar-32F	Ar-32R	Ar-33F	Ar-33R	Ar-34F	Ar-34R	Ar-36F	Ar-36R	Ar-38F	Ar-38R
E	Ar-39/57F	Ar-39/57R	Ar-41F	Ar-41R	Ar-42F	Ar-42R	Ar-45F	Ar-45R	Ar-47F	Ar-47R	Ar-48F	Ar-48R
F	Ar-49F	Ar-49R	Ar-50F	Ar-50R	Ar-51F	Ar-51R	Ar-54F	Ar-54R	Ar-55F	Ar-55R	Ar-56F	Ar-56R
G	Ar-58F	Ar-58R	Ar-59F	Ar-59R	Ar-60F	Ar-60R						
H												

PLATE 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	H-33F	H-33R	H-37F	H-37R	H-39F	H-39R	H-41F	H-41R	H-42F	H-42R	H-43F	H-43R
B	H-49F	H-49R	H-52F	H-52R	H-54F	H-54R	H-55F	H-55R	H-56F	H-56R	H-57F	H-57R
C	H-58F	H-58R	H-60F	H-60R								

WADA Delta Plate Result Averages (rhGH minus hGH Values)

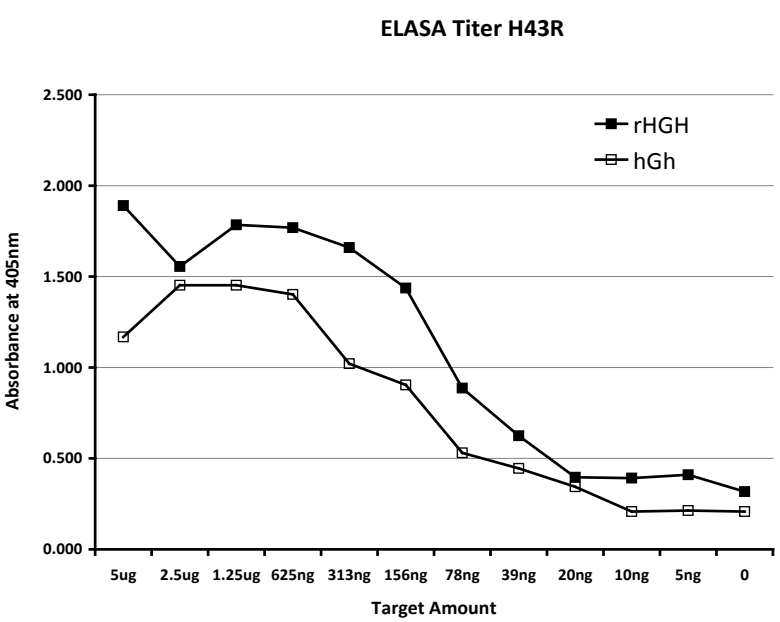
PLATE 1

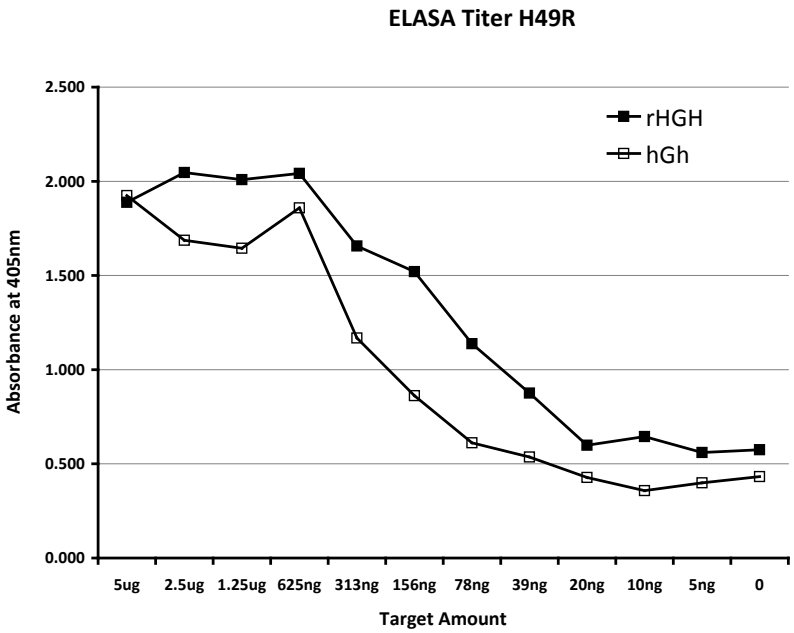
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.278	0.392	-0.103	-0.078	-0.403	-0.012	0.269	-0.339	-0.036	0.131	0.164	-0.282
B	-0.008	-0.093	0.132	0.037	-0.180	0.030	0.152	-0.016	-0.008	-0.150	-0.079	-0.141
C	0.520	0.125	-0.033	-0.033	0.467	-0.039	-0.333	0.033	-0.428	-0.318	0.247	0.292
D	0.043	0.647	0.061	0.080	0.244	0.025	-0.037	0.052	-0.078	0.425	-0.192	0.186
E	0.462	-0.025	0.026	0.145	-0.213	-0.033	0.087	-0.020	-0.037	0.019	-0.142	-0.256
F	0.217	-0.372	-0.191	0.167	0.409	0.232	0.004	0.091	-0.103	0.002	0.056	-0.037
G	0.076	-0.106	-0.330	-0.024	0.049	-0.039						
H												

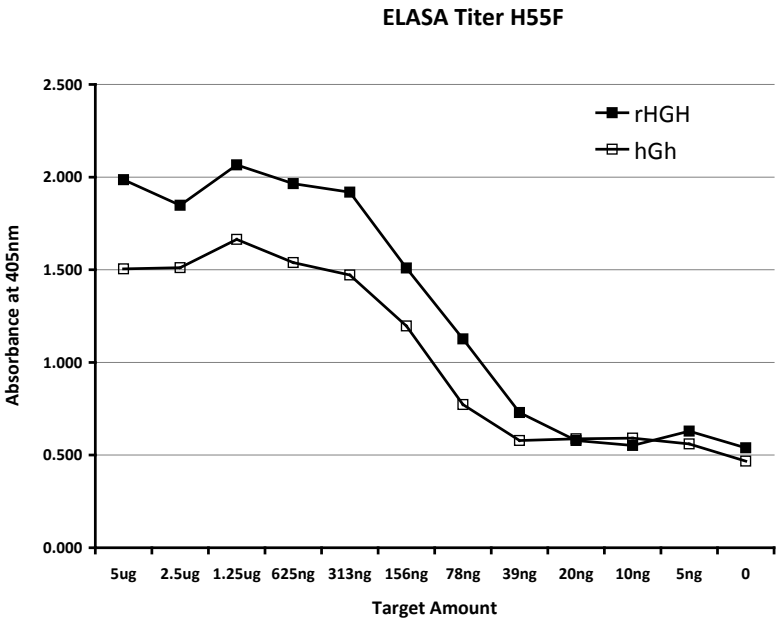
PLATE 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.281	0.271	0.127	0.196	0.027	0.223	0.418	0.437	0.322	0.343	0.337	0.512
B	0.278	0.482	0.343	0.227	0.382	0.356	0.486	0.360	0.513	0.462	0.467	0.501
C	0.273	0.282	0.161	0.259								

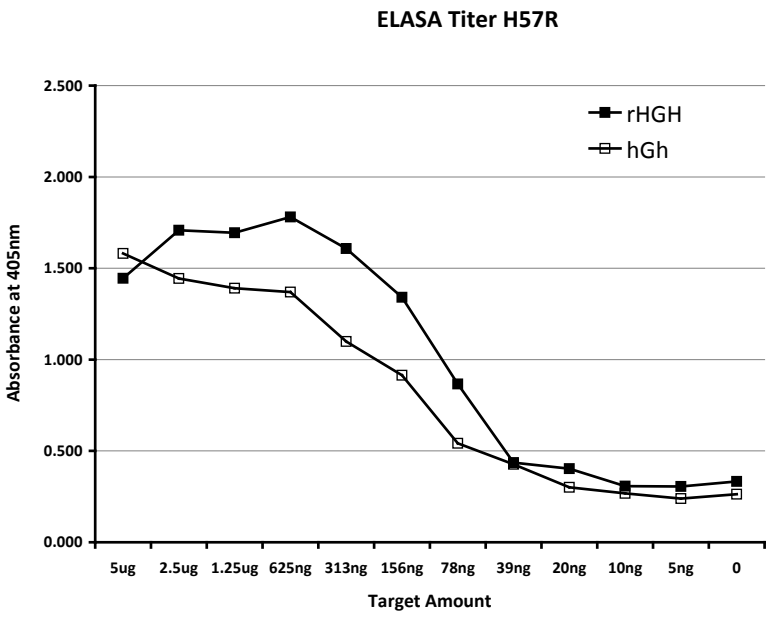
ELASA Titrations: Part 1

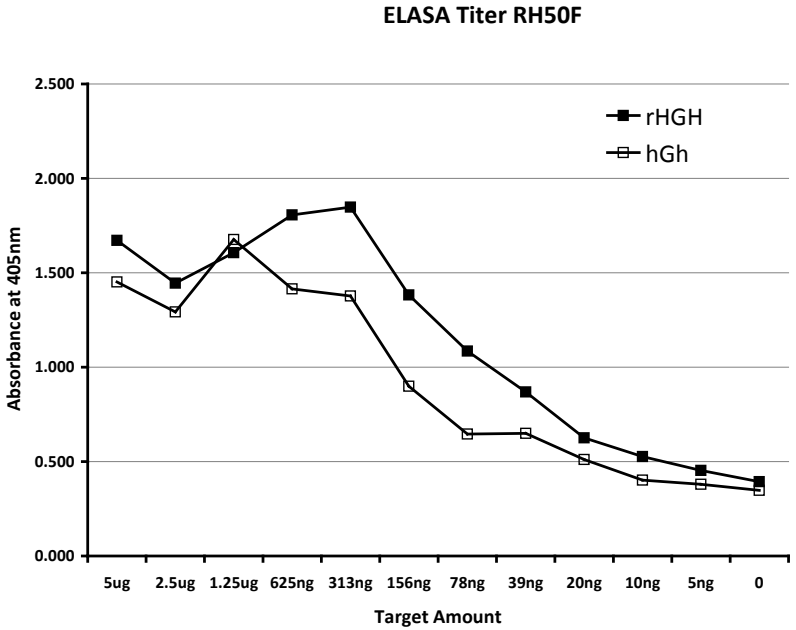


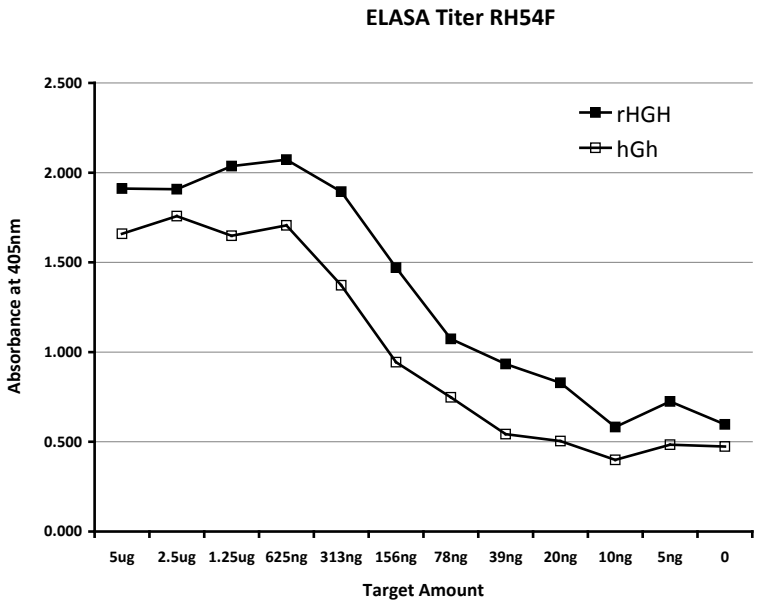


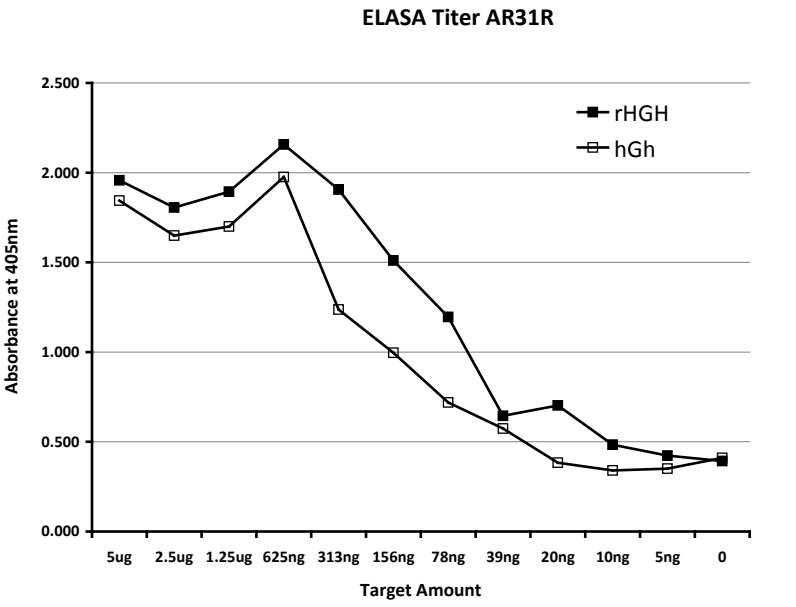


ELASA Titrations: Part 2

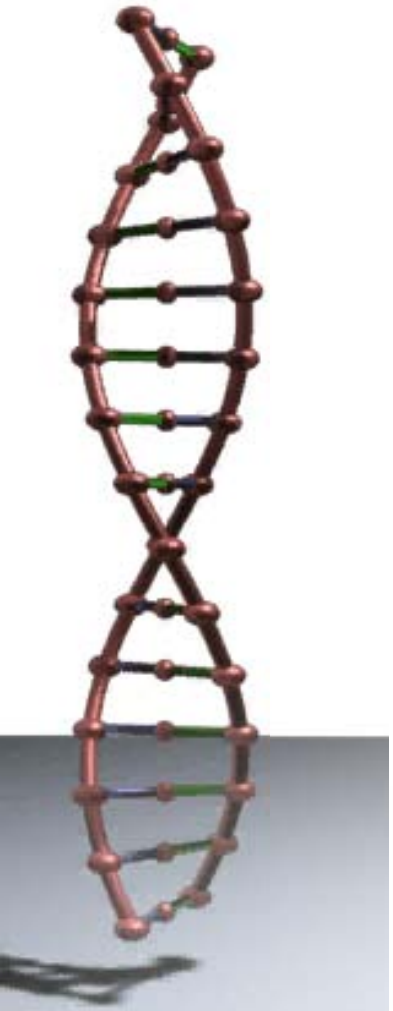


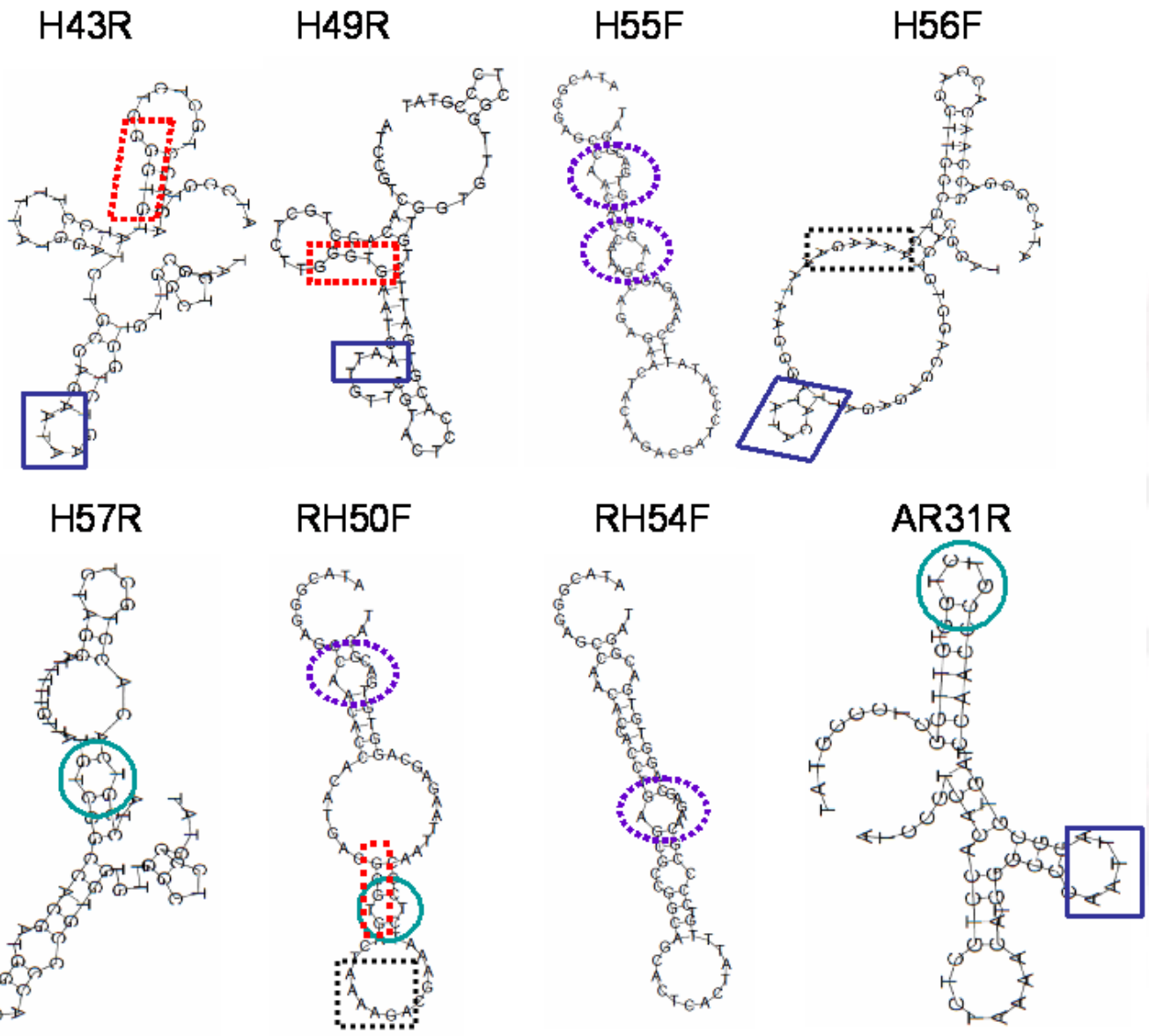




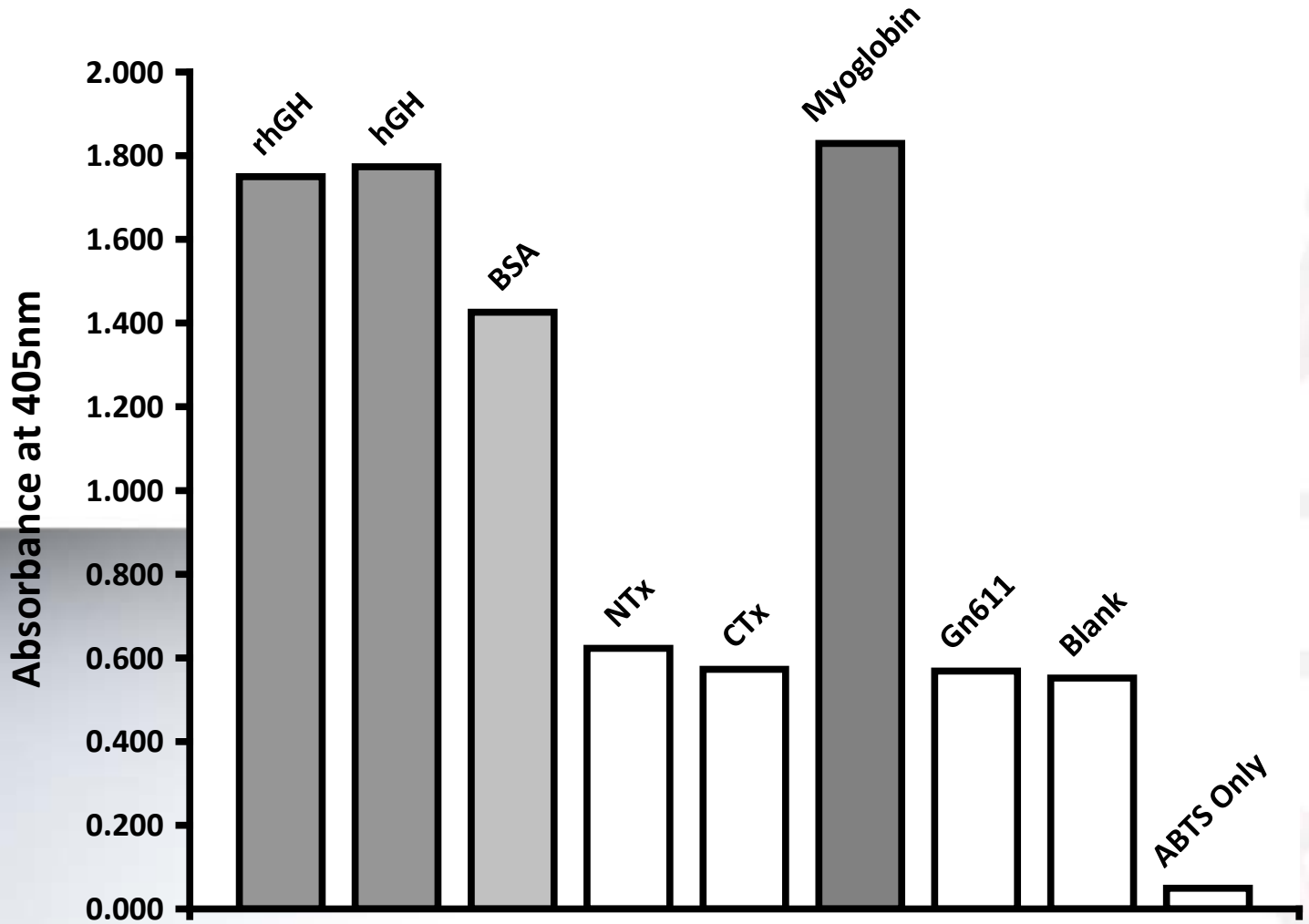


Secondary Structural Analysis





Cross-Reactivity: Means of Top 8 Aptamers



Recombinant ~~and Natural~~ hGH (GenWay Biotech)

MFPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFS
ESIPTPSNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYD
LLKDLEEGIQTLMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFR
KDMDKVETFLRIVQCRSVEGSCGF

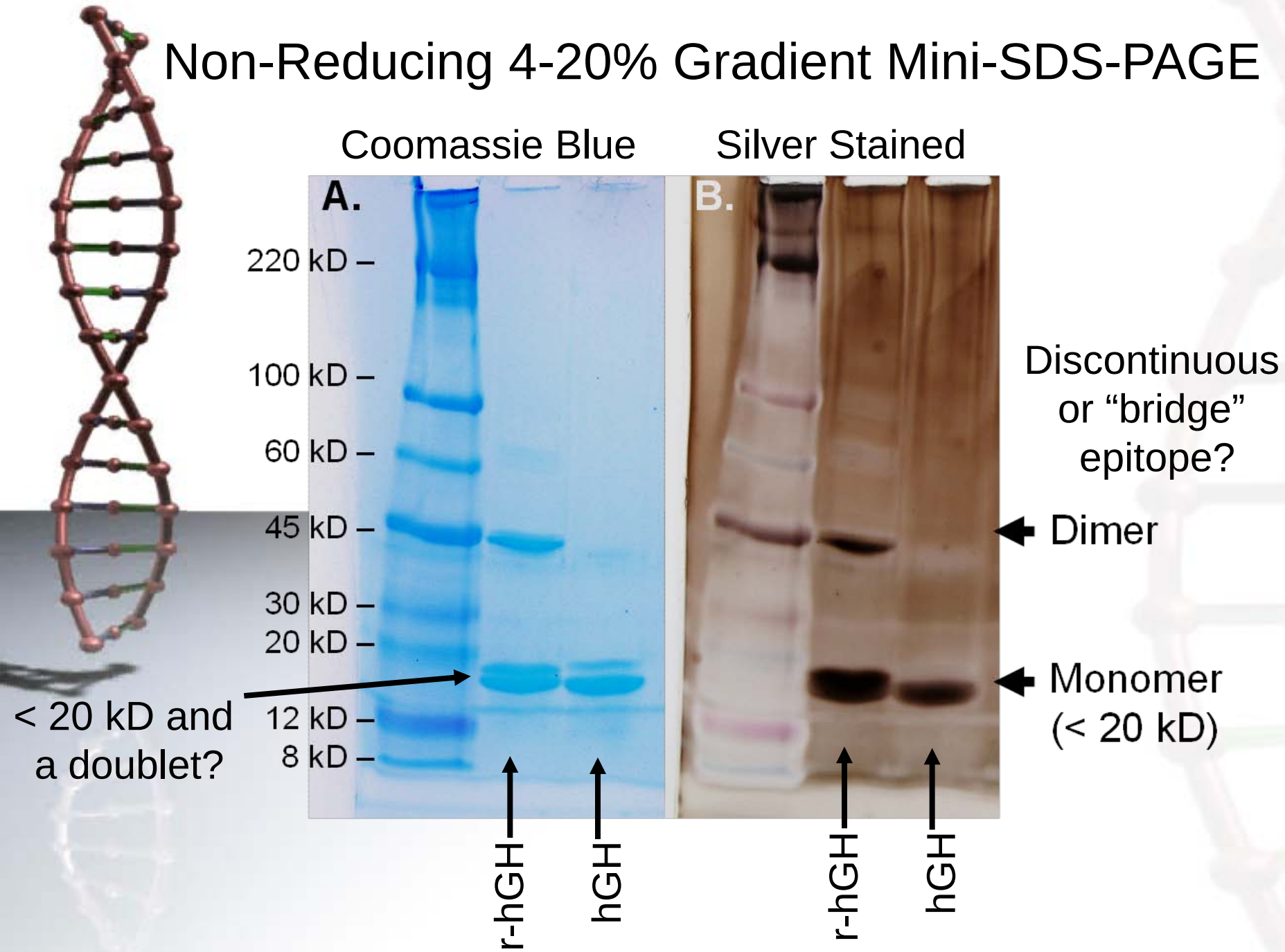
Myoglobin

MGLSDGEWQLVLNVWGKVEADIPGHGQEVLIRLFKGHPETLEKFDKFKHLKSE
DEMKASEDLKKHGATVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPVKYLEFI
SECIIQVLQSKHPGDFGADAQGAMNKALELFRKDMASNYKELGFQG

Bovine Serum Albumin

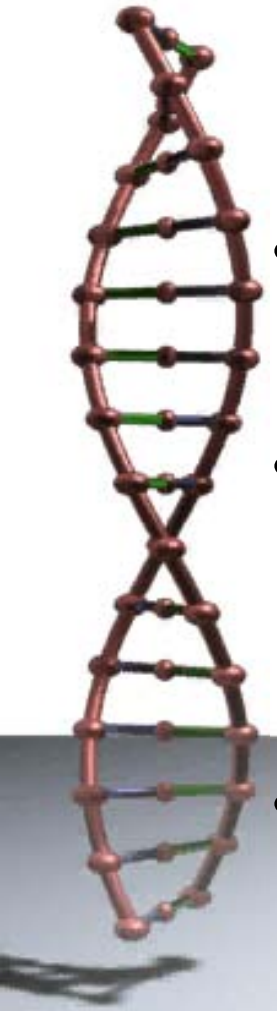
MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQYL
QQCPFDEHVKLVNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLRETYG
DMADCCEKQEPERNECFLSHKDDSPDLPKLKPDPNTLCDEFKADEKKFWGKYL
YEIARRHPYFYAPELLYYANKYNGVFQECCQAEDKGACLLPKIETMREKVLTSS
ARQRLRCASIQKFGERALKAWSVARLSQKFPKAEFVEVTKLVTDLTKVHKECCH
GDLLECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSHCIAEVEKDAIPENLP
PLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSRRHPEYAVSVLLRLAKEYEATL
EECCAKDDPHACYSTVFDKLKHLVDEPQNLIKQNCDQFEKLGEYGFQNALIVRY
TRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTEDYLSLILNRLCVLHEK
TPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKAFDEKLFTFHADICTLPDTEKQ
IKKQTALVELLKHKPKATEEQLKTVMENFVAFVDKCCAADDKEACFAVEGPKL
VVSTQTALA

Non-Reducing 4-20% Gradient Mini-SDS-PAGE



Summary of Pilot Study & Future?

- Cloned and sequenced 106 candidate hGH, r-hGH or absorbed DNA aptamers
- 8/106 aptamers gave $\Delta \geq 0.5$ A 405nm or bound consistently better to r-hGH (i.e., discriminated recombinant-hGH)
- The top 8 discriminatory aptamers shared some short sequence segments in loops
- Aptamers were made to research grade r-hGH which has an N-terminal methionine, but no 6x His tail or other affinity capture tail
- Aptamers could be detecting a discontinuous or “bridge” epitope on the dimer of r-hGH?
- Wish to target pharmaceutical r-hGH (Genotropin & Norditropin, etc.) using same r-hGH aptamer adsorption methods and known modified epitopes from Hepner et al. 2005 & 2006 in future work
- ***The ultimate aptamer-based solution would require specific epitope mapping to develop one or more highly r-hGH-specific aptamer assays.***



WADA's Critique of Pilot Study & Solutions

- ELASA not sensitive enough – Solution: ECL with sub-picogram/mL LODs
- R-hGH aptamers cross-reacted with BSA & myoglobin – Solution: Adsorb r-hGH aptamers with various human serum proteins including albumins, Hb & Mb to increase selectivity
- Many pharmaceutical r-hGH products would require vast amount of work to restart aptamer development for each – Solution: Find common host cell modifications from Hepner et al. in Genotropin and Norditropin and target those "epitopes" as initial aptamer development targets (Next slide)

Hepner et al., 2005/06 – Common *E. coli* modifications to Target?

Proteome Science 2005, **3**:1 http://www.proteomesci.com/content/3/1/1

Table 2: Results of electrospray LC-MS/MS and MALDI-TOF MS/MS measurements

Sequence	M calculated (Da)	M observed (Da)	delta M (Da)	Modification
EETQQ**K**SNLELLR	1586.83	1614.74	27.91	K_{70}: di-methylation or → R *
FDT**N**SH**N**DDALLK	1488.68	1489.60	0.92	N_{149} / N_{152} deamidation
RLEDGSPR	928.47	900.46	-28.01	R_{127}→ Q or K *
LFDNA**M**LR	978.50	994.40	15.90	M_{14} : oxidation
D**M**DKVETFLR	1252.61	1268.40	15.79	M_{170}: oxidation
DLEEGIQTL**M**GR	1360.61	1376.66	16.05	M_{125}: oxidation
LFDNA**M**LR	978.50	960.40	-18.10	M_{14} → I
DLEEGIQTL**M**GR	1360.61	1342.60	-18.01	M_{125} → I
D**M**DKVETFLR	1252.61	1234.60	-18.01	M_{170} → I
LFDNAMLR	978.50	1035.40	56.90	Carbamidomethyl – N terminus

• Nearly isobaric mass differences

Sequences and mass differences of modified peptides detected in the Genotropin sample. Column 1: sequence of modified peptides following tryptic digestion of Genotropin®; column 2: calculated monoisotopic masses of the unmodified tryptic peptides; column 3: observed monoisotopic masses; column 4: differences of calculated and observed masses; column 5: possible explanations of the mass differences.

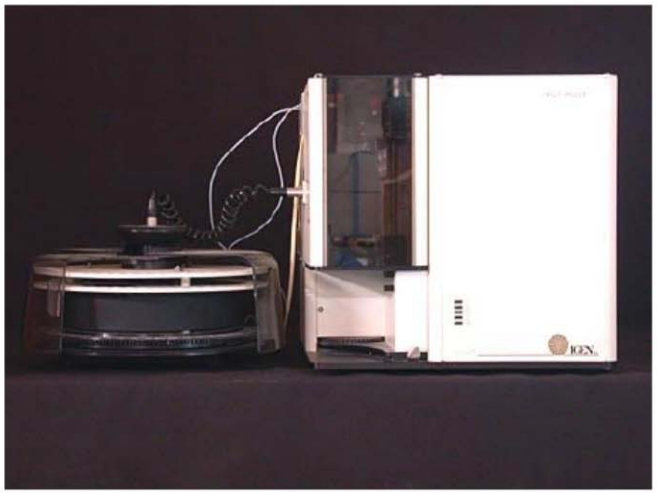
Modifications to approx. 2% of Genotropin made in *E. coli* K12

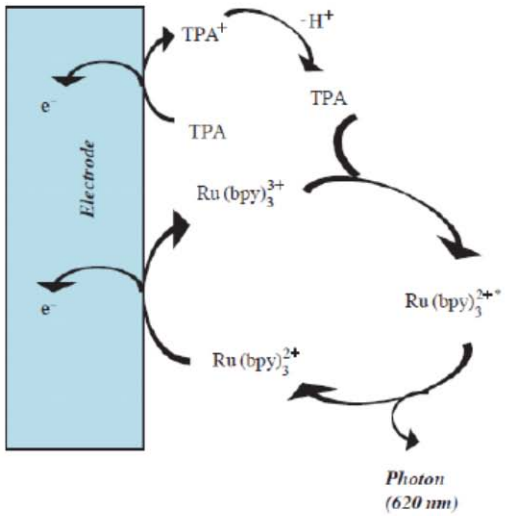
Hepner et al., 2006: Norditropin M14 → V14

Ultrasensitive Electrochemiluminescence (ECL)

IGEN / Bioveris – Defunct
Research ECL Analyzer

Origen®
Analyzer

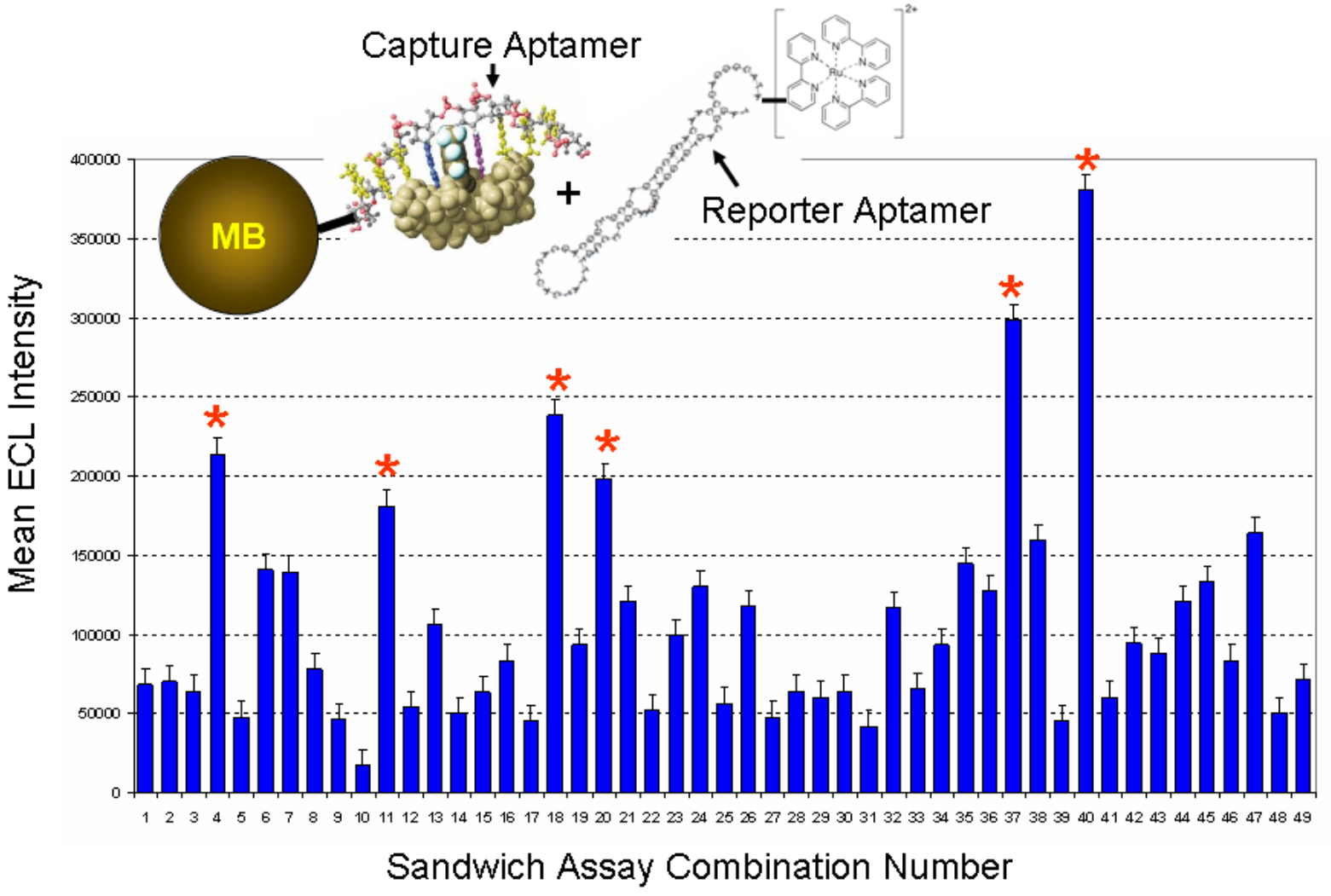




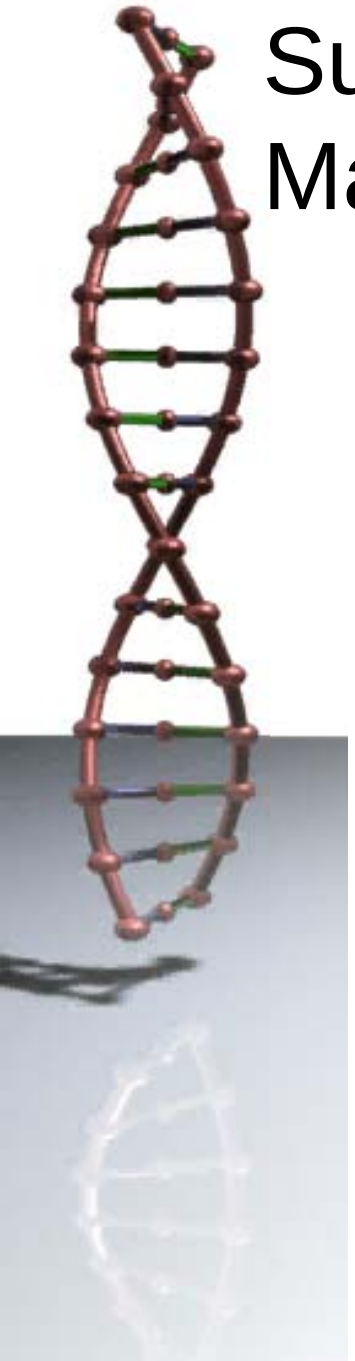
Roche ELECSys Clinical ECL Analysis Systems

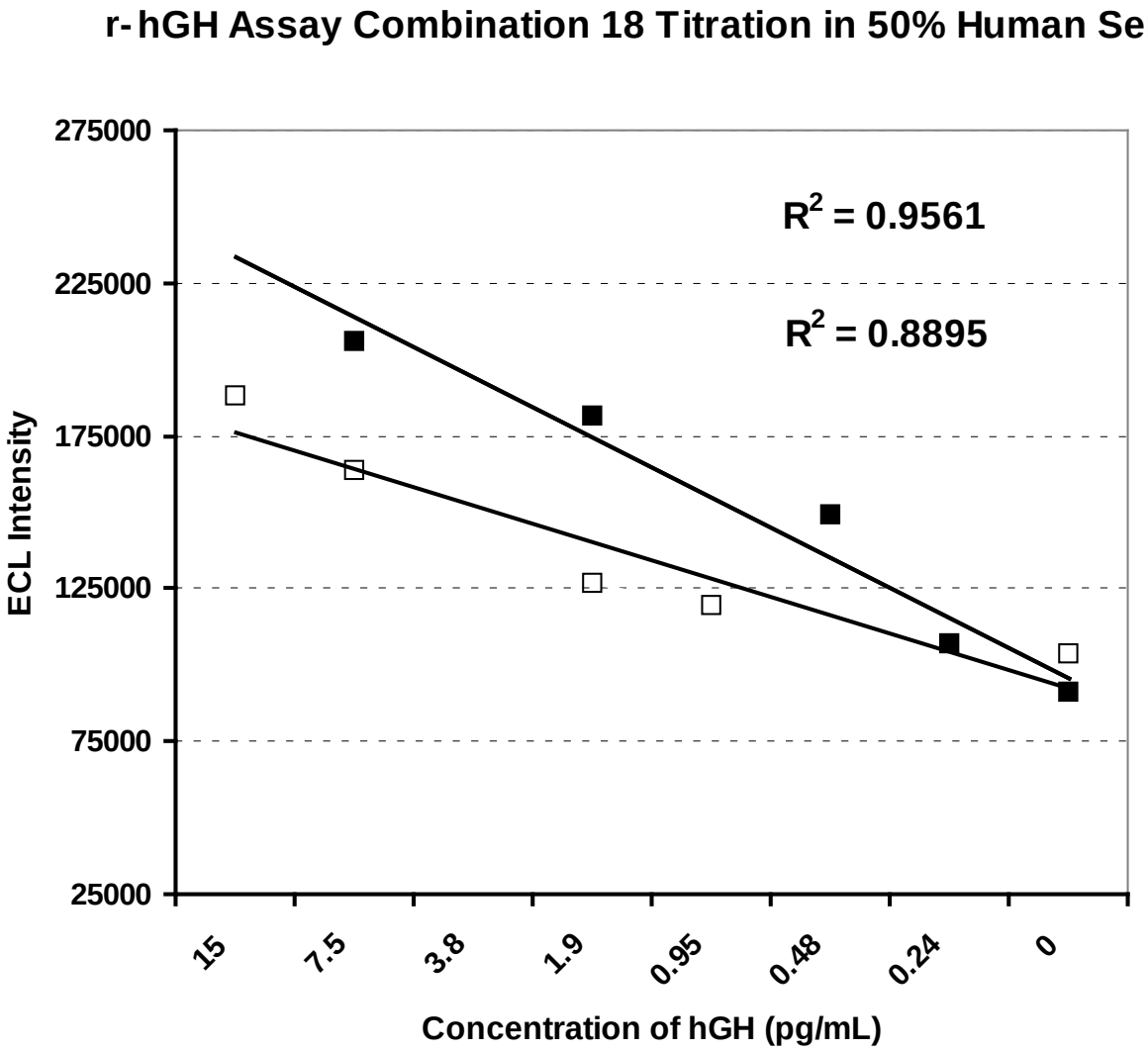
Roche
Cobas e 411

ECL Sandwich Screening Results

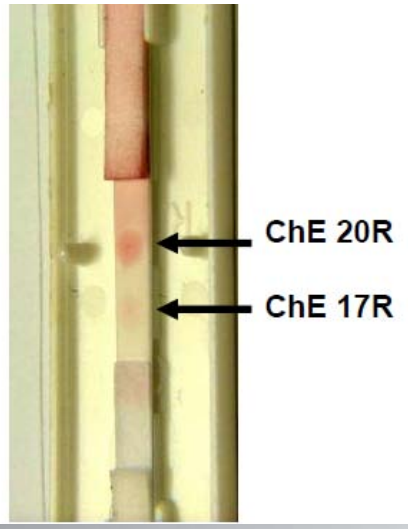


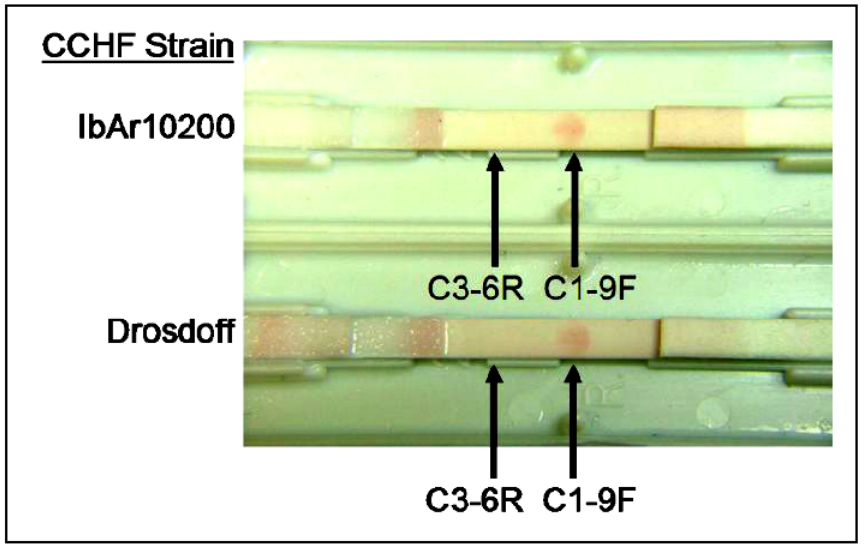
Sub-pg hGH detection by Aptamer-Magnetic Bead ECL Sandwich Assay



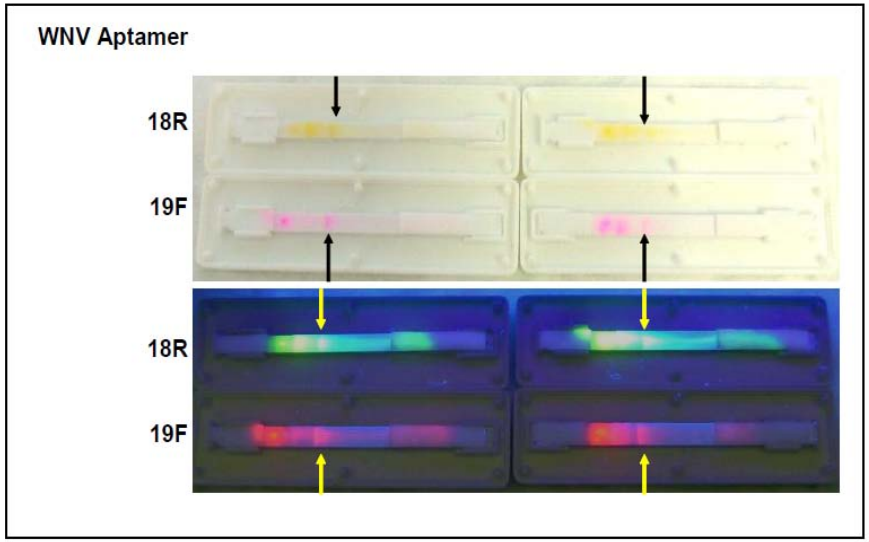


Presumptive Lateral Flow Aptamer Strips for On Site r-hGH Testing?

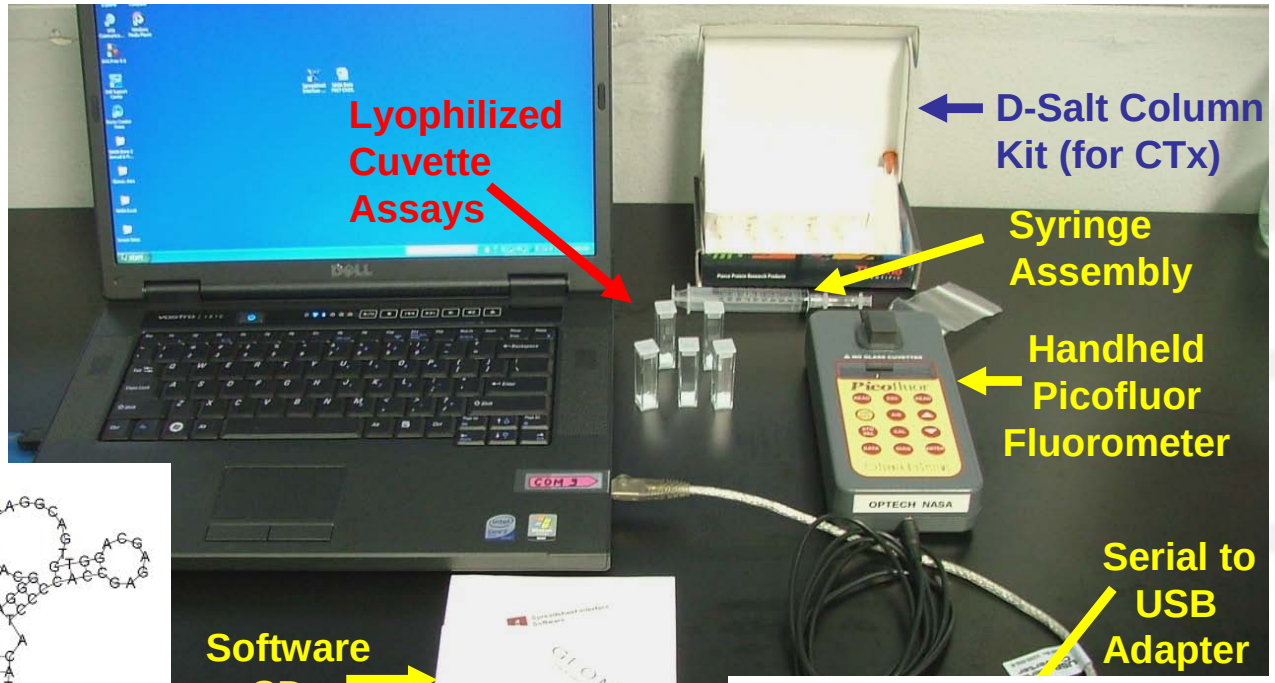


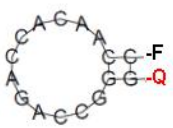


- Traditional colloidal gold
- Enhanced sensitivity w/ fluorescent NPs or Q-dots and a UV penlight

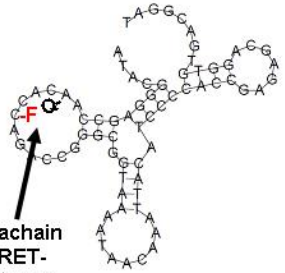


Rapid sensitive on site FRET-aptamer assays for r-hGH?

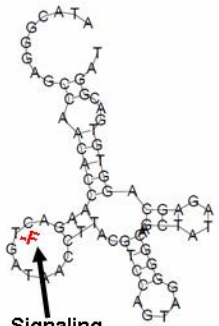




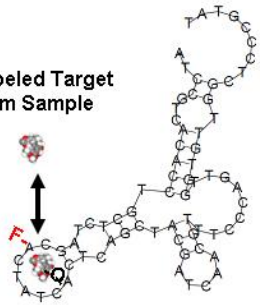
Molecular (Aptamer) Beacon



Intrachain FRET-Aptamer

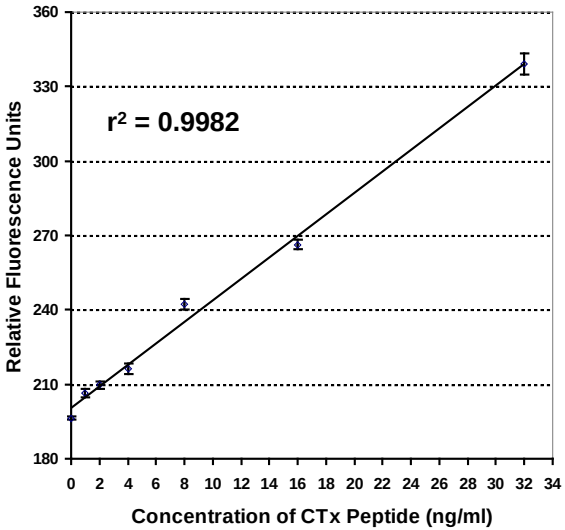


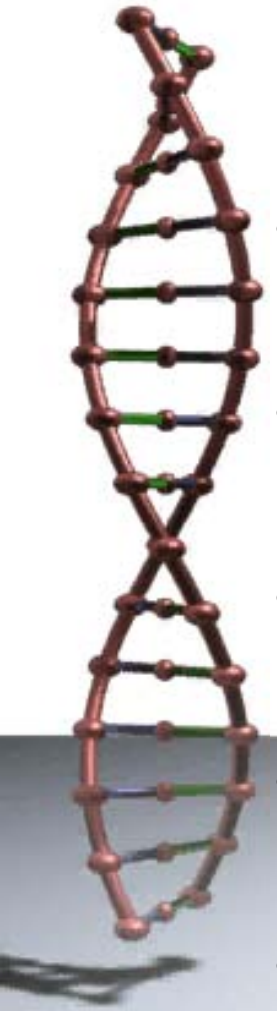
Signaling Aptamer (No Q Incorporated)



Competitive FRET-Aptamer

Handheld Bone Peptide Sensor Delivered to NASA Phase 2 SBIR





Key References

- Andersson C., et al. Isolation and characterization of a trisulfide variant of recombinant human growth hormone formed during expression in *Escherichia coli*. Int. J. Pept. Protein Res. 47:311-321, 1996.
- Bruno J.G., et al. Discrimination of recombinant from natural human growth hormone using DNA aptamers. J. Biomolec. Techniques. 22:27-36, 2011. (Free on Internet at JBMT website & PubMed)
- Bruno J.G. and Kiel J.L. Use of magnetic beads in selection and detection of biotoxin aptamers by ECL and enzymatic methods. BioTechniques. 32:178-183, 2002.
- Hepner F., et al. Mass spectrometrical analysis of recombinant human growth hormone (Genotropin) reveals amino acid substitution in 2% of the expressed protein. Proteome Sci. 3:1-12, 2005.
- Hepner F., et al. Mass spectrometrical analysis of recombinant human growth hormone Norditropin reveals amino acid exchange at M14-V14 r-hGH. Proteomics 6:775-784, 2006.
- Lispi M., et al. Heterogeneity of commercial recombinant human growth hormone (r-hGH) preparations containing a thioether variant. J. Pharm. Sci. 98-4511-4524, 2009.