Translation Fidelity Rate Determination in a Cell-Free Protein Synthesis System using LC-MS

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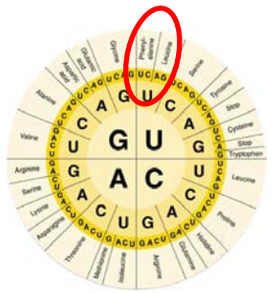
Overview:

- LC-MS is used to determine the site-specific rate of translation fidelity
- The Phe→Leu mis-translation event was specifically investigated
- Addition of streptomycin increases the amount of mis-incorporation[1]
- Rates of mis-incorporation measured are higher for *in vivo* vs. *in vitro* product

Introduction:

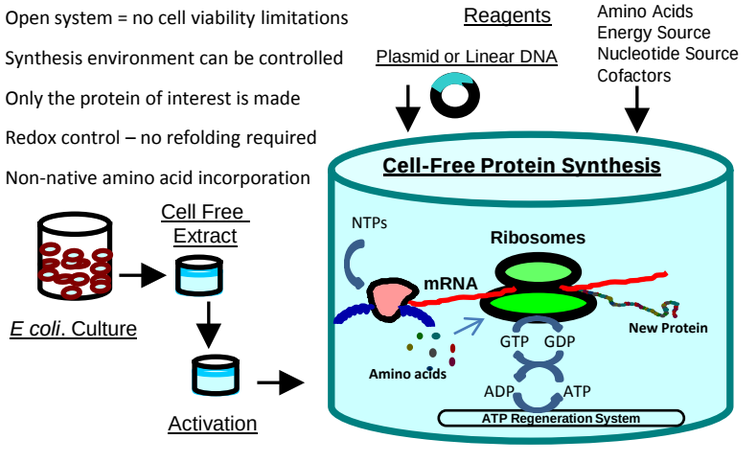
There is always some amount of mis-incorporation during protein synthesis at an associated translation fidelity or mis-incorporation rate. Current methods determine this rate using genetic or radiological assays, but they do not examine the protein sequence directly and can only be applied to one mis-incorporation event at a time. Use of LC-MS allows residue-specific translation fidelity determination. Phe→Leu mis-incorporation was the focus in this study. The codons for these amino acids are the same except for the third or "wobble" position. The last position has less selectivity and is more prone to mis-pairing during translation resulting in an undesired amino acid beinginserted into the protein.



Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) was used as the test protein to evaluate translation fidelity of the Sutro cell-free synthesis system. We have previously shown that this protein is produced fully soluble and properly folded with correct disulfide formation for up to a 100 L reaction.

What is Cell-Free Synthesis?

- Sutro Biopharma has developed a cell-free or *in vitro* transcription/translation platform for protein expression in the absence of living cells.
- Open system = no cell viability limitations
- Synthesis environment can be controlled
- Only the protein of interest is made
- Redox control – no refolding required
- Non-native amino acid incorporation



References:

1. Szaflarsk W., et al. *J. Mol. Bio*. **380**, 193–205 (2008)
2. Wang L. , et al. *Proc. Nat. Acac. Sci. USA*. **100**, 56–61 (2003)

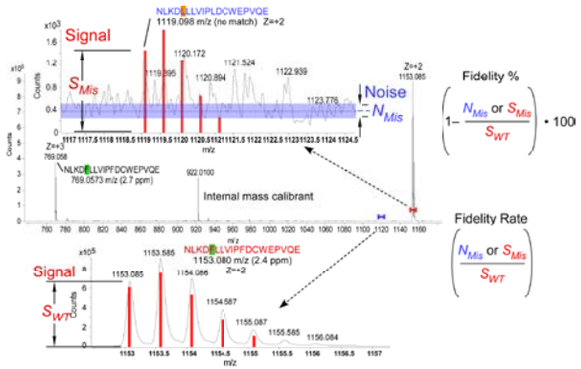
Methods:

Sample preparation:

After disulfide reduction with tris(2-carboxyethyl)phosphine HCl (2mM in NH_3COCH_3 pH 6.5, 30 min at 37 C) rhGM-CSF was digested with Glu-C protease (1:20 protease:substrate) for 15 h at 37 C. Samples were centrifuged at 14,000 g for 15 min. Protein digests were analyzed on an Agilent 6520 Accurate Mass Q-TOF LC-MS system equipped with a nano-electrospray ChipCube source fitted with a Zorbax 300SBC18 HPLC Chip (analytical column: 5 µM particle size, 43 mm 75 µm, enrichment column: 4mm, 40 nL) and eluted by a linear acetonitrile gradient (0–90%, 0.1% formic acid).

Data analysis:

An entire chromatogram was searched for reasonable digested peptides that may contain a Phe to Leu mis-incorporation along with their wild-type analogs. Peptides were located using extracted ion chromatograms (EICs) centered on the peptide theoretical m/z within a reasonable mass error (20 ppm for these experiments). This is a reliable method when using accurate mass MS along with the peptide sequence verified by tandem MS. For this presentation the ion signal was quantified by summing the intensity of all monoisotopic ions over the entire elution profile of the peptide of interest.

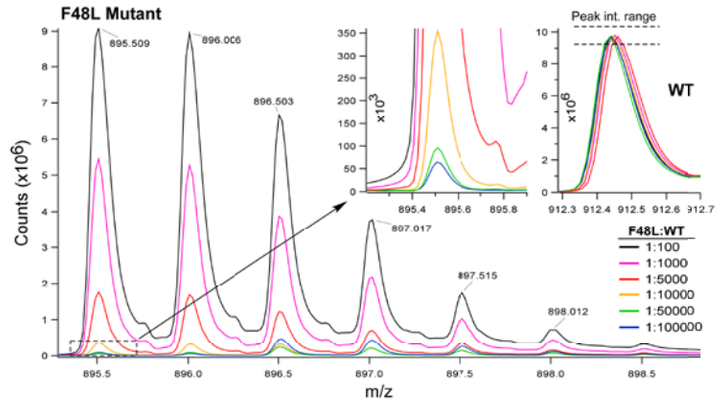


In absence of a peptide resulting from mis-incorporation, its signal was assumed to be equal or less than the noise in the mass spectrum. The resulting minimum fidelity rate was calculated as a signal-to-noise ratio (S/N) at the region in the mass axis expected to contain the peptide with mis-incorporated Leu.[2]

Results:

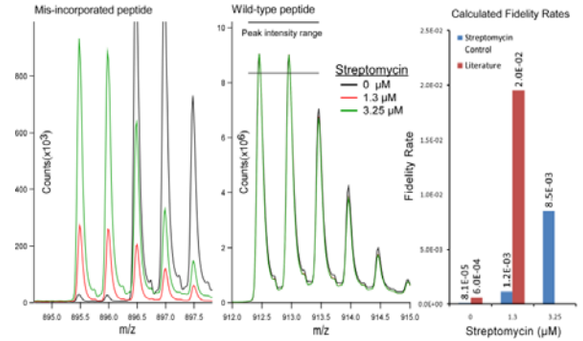
Wild-type and mutant protein titration:

rhGM-CSF wild-type (WT) protein was mixed with a decreasing amount of a F48L mutant and digested. The peptides corresponding to the mutant and WT proteins were tracked to gauge the dynamic range of the method and calibrate the signal calculation method. Since the same amount of WT protein was used for each sample, all mutant spectra were normalized on the WT peptide signal intensity. The peptide of the opposing species was confirmed to be absent in a separate digest of each protein.



Decrease in translation fidelity with addition of streptomycin:

Addition of aminoglycoside antibiotics is known to increase the amount of mis-incorporation during transcription.[1] Streptomycin was added to a standard cell free reaction at 0 (control), 1.3, and 3.25 µM. Both wild-type and the mis-incorporated peptides were observed in all samples and the amount of mis-incorporated peptide increased with the streptomycin concentration. Peptides corresponded to those observed in the rhGM-CSF wild-type and F48L mutants, and were confirmed by tandem MS. All peptide ion intensities were normalized to the wild-type peptide.



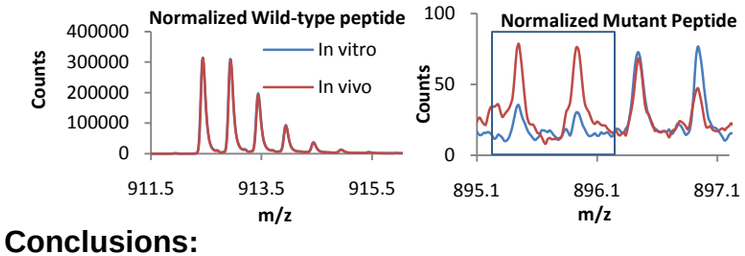
Fidelity rate determination of wild-type rhGM-CSF from a 100 L cell-free reaction:

rhGM-CSF was produced at 100 L scale. All rates were calculated based on the S/N except for the peptide covering position F48. For this position a small signal corresponding to the mis-incorporated peptide in mass and elution time was found (no MS/MS could be obtained). As a conservative estimate the intensity of this ion was used instead of the S/N of the wild-type peptide to estimate the rate of fidelity for this position.

Phe Position	Sequence	S/N	Est. Fidelity Rate	Est. Fidelity %
104	TSCATQIITFE	8.12 E+04	1.23E-05	99.999
48	MFDLQEPTCLQTRLE	2.49E+05	9.89E-05	99.990
107,114,120	SFKENLKDFLLVIPFDCWEPVQE	1.08E+04	9.25E-05	99.991

***In vitro* fidelity is better than *in vivo*:**

Comparison of *in vitro* and commercial *in vivo* synthesized rhGM-CSF shows that the translation fidelity is better for the *in vitro* product (blue) that shows less misincorporated peptide. The spectra below are normalized on the average intensity of the monoisotopic wild-type peptide ion. Fidelity rates are estimated to be 10^{-5} for both samples.



Conclusions:

- Translation fidelity can be measured site-specifically with LC-MS
- The method has a five order of magnitude dynamic range
- Protein synthesis in Sutro's cell-free system has greater translation fidelity compared to *in vivo* systems.
- Ion intensity measurement methods are being further explored