Investigating Stability of Multiple Forms of Interferon-Gamma Inducible Protein 10 in Plasma Using a Novel 3-Plex Immunoassay

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Summary

Interferon-gamma Inducible Protein 10 (IP-10), also known as CXCL-10, is a proinflammatory chemokine that attracts monocytes, T-lymphocytes, and natural killer cells. IP-10 induction in several different disorders including autoimmune diseases, kidney injury, cancer, and infectious disease is currently being studied. Hepatitis C virus (HCV) infection is of particular interest to researchers because more than half of infected adults develop chronic infections, and nearly half of those progressing to chronic infection do not respond to standard treatment. It has been noted in studies that higher levels of IP-10 correlate to "non-responder" status in patients [1]. This is counterintuitive because of the chemoattractive properties of IP-10 for activated immune cells. Recent work by Casrouge, *et. al.* [2,3], showed that the increase in IP-10 concentration in non-responding patients was due to an increase in a specific alternative form of IP-10 which has an antagonist activity. This form is generated by dipeptidyl peptidase – 4 (DPP-4) which cleaves the first two amino acids off of the full length IP-10. Therefore, in order to better predict responder versus non-responder status for treatment, it is important to distinguish the DPP-4 cleaved (short) form from the full length (long) form. Equally as important is the preservation of the different forms of IP-10 for the period between sample collection and analysis. DPP-4 is known to be active in blood and can continue to generate short IP-10 at the expense of long IP-10 even after the sample is collected unless properly managed.

The following work addresses the need for a means to distinguish between these forms of IP-10 with opposing activities and maintain their respective concentrations prior to analysis. First, a multiplex immunoassay was developed by Myriad RBM with epitope specificity capable of simultaneously measuring the total, long, and short forms of IP-10 with minimal cross-reactivity within the IP-10 3-PLEX Multi-Analyte Profile. Second, stability of recombinant IP-10 (rIP-10) spiked into human plasma during room temperature incubation was investigated using the aforementioned immunoassay and evacuated blood collection tubes containing protease inhibitors including a DPP-4 inhibitor.

The results showed that the assay was specific for the long and short forms of IP-10. Also, the standard BD™ EDTA tube showed generation of short IP-10 over time indicating DPP-4 activity while the BD™ P700* protease inhibitor containing tube prevented it. Additionally, the BD™ P700 tube preserved a significantly larger portion of the spiked long IP-10 even at the point of collection compared to the EDTA control.

Methods

DPP-4 Digest

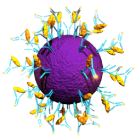
- A 100 µg/mL stock of rIP-10 was diluted 2x with 1xTE buffer pH 7.65 and 0.4 µU DPP-4.
- The full length control was prepared the same way without adding DPP-4.
- The solutions were incubated for 8 hours at 37 °C.
- Control and digest samples were mixed with MALDI matrix (50% ACN, 0.1% TFA, 5 mg/mL CHCA) and spotted on a MALDI target.
- Mass spectra recorded with an Ultraflex II MALDI-TOF MS (Bruker-Daltonics) in linear mode.

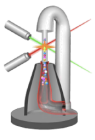
IP-10 Plasma Time-course

- Venous blood was collected from three healthy, adult volunteers into BDTM K2EDTA, and BDTM P700* (protease and DPP-4 inhibitor) evacuated blood collection tubes.
- Tubes were immediately centrifuged at 2,500 x g for 20 minutes.
- Plasma was aliquotted and spiked to 10,000 pg/mL full-length rIP-10 (PeproTech).
- Spiked plasma was incubated at room temperature for the duration of the time-course.
- At specified times, plasma aliquots were removed, acidified to 0.1% TFA for (Subjects 2 and 3 only), and frozen at -80 °C (all subjects).
- Long and short controls diluted in animal serum (R&D Systems) and frozen at -80 °C.
- Samples shipped to Myriad RBM on dry ice for analysis with IP-10 MAP 3-PLEX kit.

Multiplex Immunoassay

- Samples were analyzed using Myriad RBM's IP-10 MAP, a microsphere-based 3-plex immunoassay that measures the total IP-10 protein in addition to the short and long forms of the protein.
- Assays were run on an automated Luminex xMAP™ platform at the Myriad RBM CLIA certified lab (Austin, TX) and have been validated for the fundamental assay parameters of least detectable dose (LDD), lower limit of quantitation (LLOQ), spike recovery, linearity, precision and sample stability. Full assay validation documents can be requested from Myriad RBM (www.myriadrbm.com)
- During the automated run, high concentration standards (loaded during the sample plating) are serially diluted to produce two sets of eight-point calibrators that incorporate every analyte in the multiplex. After the plate has been read, this dual set of standard concentration values is fitted using a set of proprietary curve-fitting routines. The algorithms use four- and five- parameter equations to produce the best description of the standard values. The MFI for each sample is then plotted on the standard curve and the final concentration is derived by multiplying by the sample dilution factor. In addition, all assays have unique sets of three-level controls (high, medium, and low concentration) for each of the analytes within the multiplex.





*BD P700 tubes for Research Use Only, Not for Diagnostics Purpose

Results – DPP-4 Digestion of rIP-10

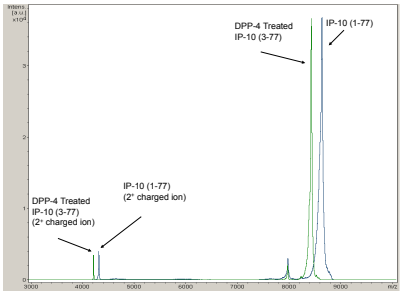


Figure 1 - Mass spectra of a DPP-4 digest of rIP-10 in buffer (green) and the control sample without DPP-4 (blue).

Table 1 – Immunoassay results for rIP-10 stock (used for spiking) and IP-10 DPP-4 digest in animal serum.

	total	long	short
Long ctrl (pg/mL)	10600.0	10045.0	3060.0
stdev	565.7	219.2	56.6
Short ctrl (pg/mL)	11900.0	<LDD	14800.0
stdev	0.0	N/A	0.0

- rIP-10 sequence (1-77)

  VPLSRTVRCTCISISNQPVNPRSLEKLEIIPA SQFCPRVEIIATMKKKGEKRCLNPESKAIK NLLKAVSKERSKRSP[3]

  - Sequence has preferred DPP-4 cleavage site (X-Pro)
- Confirmed DPP-4 cleaves IP-10
- Multiplex assay specific for IP-10 long and short forms

Results – Plasma Time-course

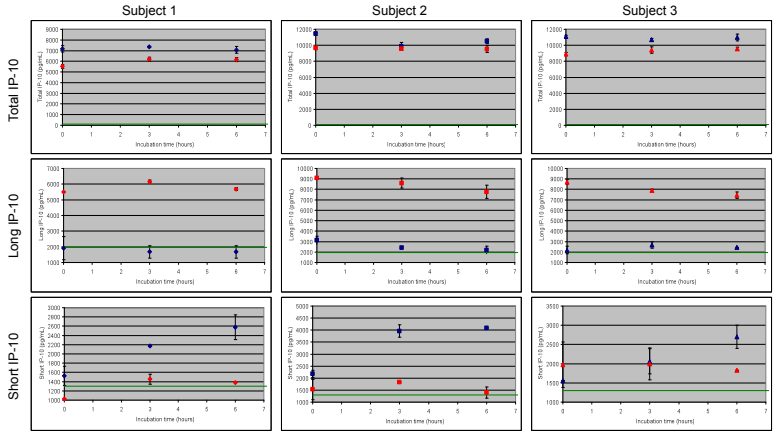


Figure 2 – Time course for different IP-10 forms in EDTA (blue) and P700 (red) plasma with room temperature incubation. Least detectable dose (LDD) for the assay shown by green line.

- Total IP-10
  - EDTA and P700 have comparable and relatively stable time courses.
- Long IP-10
  - EDTA shows very low levels even at time zero
  - P700 shows levels significantly closer to spiked levels at time zero with a slow degradation out to 6 hours.
- Short IP-10
  - EDTA shows clear increase in short IP-10 form over time
  - No increase in short IP-10 form seen in P700

Conclusions

- Myriad RBM IP-10 MAP 3-PLEX assay is specific for long and short forms of IP-10.
- P700 stabilizes the profile of long and short IP-10 forms out to 6 hours of plasma incubation at room temperature.
- Long IP-10 values in EDTA even when acidified were not representative of the amount spiked at time = 0.
- Assay was not affected by any acidification steps.

References

1. Butera D, *et. al. Blood*. **106**, 2005, 1175-82.
2. Casrouge A, *et. al. Clinical and Experimental Immunology*, **167**, 2011, 137-148.
3. Casrouge A, *et. al. Journal of Clinical Investigation*, **121** (1), 2011, 308-317.