

1. Observation and explanation of the differences of ERFE levels detected by the assay depending on the applied sample dilution

- When using crude (undiluted serum/plasma), upon EPO treatment an increase of ERFE is observed over several days (from 2ng/ml to 16 ng/ml)
- When using diluted serum/plasma (>1/150), upon EPO treatment no increase of ERFE is observed and levels of ERFE stay >300ng/ml
- An important parameter when testing ELISA Kits is the linearity. If Interfering factors compromise assay linearity, the ELISA is not valid. For our Erythroferrone (h) ELISA kit, linearity has been tested and the assay is linear from dilution >1/150.

BUT

- It is puzzling to observe, an increase of ERFE with our ELISA kit upon EPO treatment only in undiluted or low diluted (to 1/10) serum/plasma.
- A tentative explanation for the dilution effect has been postulated in the model presented in the next two pages with the following conclusions:
 - Steady-state ERFE protein should be mainly found as high molecular weight complex (HMW) in serum/plasma: dilution would disrupt the complex leaving only Erythroferrone dimers (the smallest form of ERFE) liberating more free epitopes and therefore higher values detected by the ELISA kit
 - ERFE is secreted from muscle and erythroblasts: muscle would secrete mainly the HMW form of ERFE while the erythroblasts would secrete only the dimer form of ERFE.
 - Upon EPO treatment, erythroblasts would secrete ERFE (dimer) and this increase of erythroblast ERFE would be only visible in low diluted serum/plasma.
 - The new amount of ERFE secreted by erythroblasts upon EPO treatment would be negligible compared to the total levels of ERFE molecules in serum.

Note:

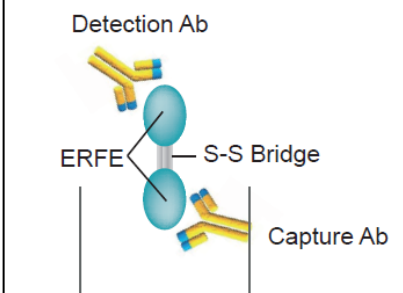
- *Erythroferrone belongs to the CTRPs family of proteins (including Adiponectin) that are found in serum/plasma at levels >100ng/ml.*
- *CTRP family members are found as monomer, dimer, trimer or multimer (>36mer for Adiponectin).*
- *Erythroferrone (mouse) levels have been measured (by quantitative western blotting) to be close to 500 ng/ml.*

WARNING: Intended for research use only. This product is not intended or approved for human, diagnostics, therapeutic or veterinary use. Use of this product for human or animal testing is extremely hazardous and may result in disease, severe injury, or death. **MATERIAL SAFETY DATA:** Review the complete Material Safety Data Sheet before use.

2. ERFE Detection: Hypothesis to explain the huge difference of ERFE levels due to Dilution

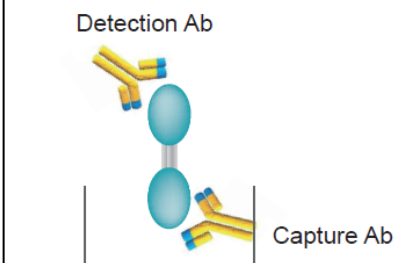
i) If ERFE is only found as S-S Dimer

ii) If ERFE is found as higher order complex (of non-covalently bound ERFE S-S-Dimer)

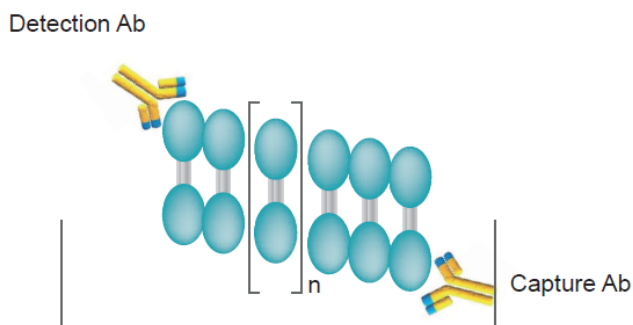


2ng/ml (hypothetical without dilution)

Dilution

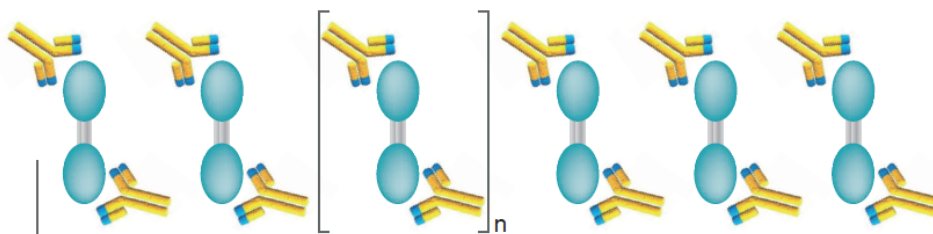


2ng/ml (hypothetical after dilution)



2ng/ml (observed levels in serum/plasma by our ELISA without dilution)

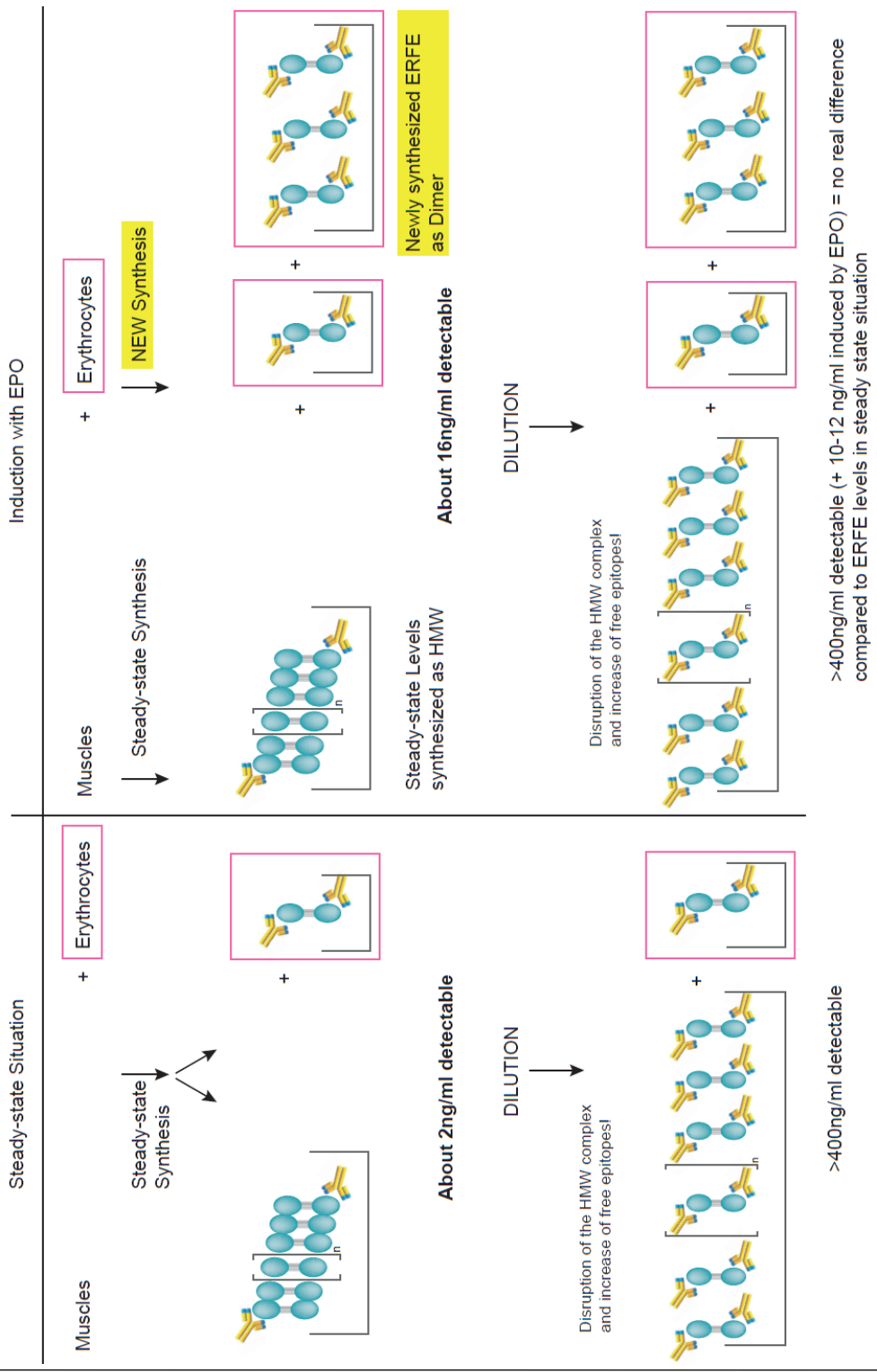
Dilution (>1/150)



>400ng/ml (observed levels in serum/plasma by our ELISA after dilution)

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3. ERFE Detection and Dilution / Steady-State vs. Induction with EPO



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4. Tips to use the Erythroferrone (human) ELISA kit (AG-45B-0014):

- To detect ERFE increase upon Erythropoietin (EPO) treatment or blood transfusion, use undiluted or low diluted serum/plasma (about 1/10):
 - Values measured will range from 2-4 ng/ml to 15-25 ng/ml.
- To detect steady state levels of ERFE, use diluted serum (>1/150).
 - Values will range from 300 ng/ml to >1200 ng/ml
- Erythroferrone has been shown to be expressed in muscle (called Myonectin/CTRP15) or in erythroblasts (called Erythroferrone /ERFE).

→ **Erythroferrone (human) ELISA Kit (#AG-45B-0014) specifically detects ERFE present in serum, plasma and cell culture supernatant.**

19-11-2019/AT

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