Sanquin

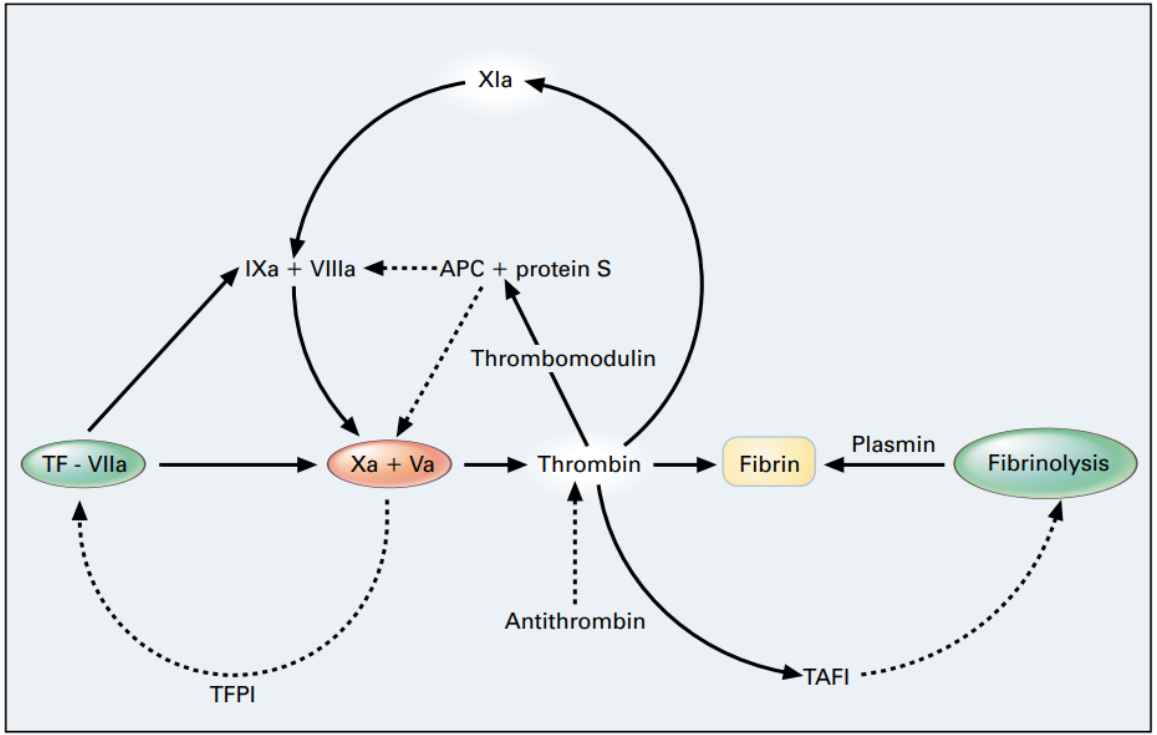
Haemostasis biomarkers

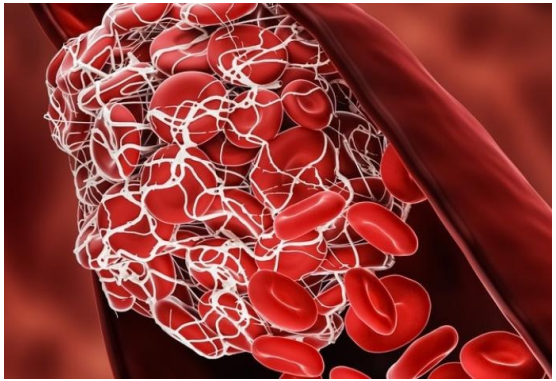
Quantitative analysis

Annick de Vries , Chief Scientific Officer, Sanquin
on behalf of Ian de Bus, PhD

For Life.

Haemostasis-
tightly regulated system of blood coagulation



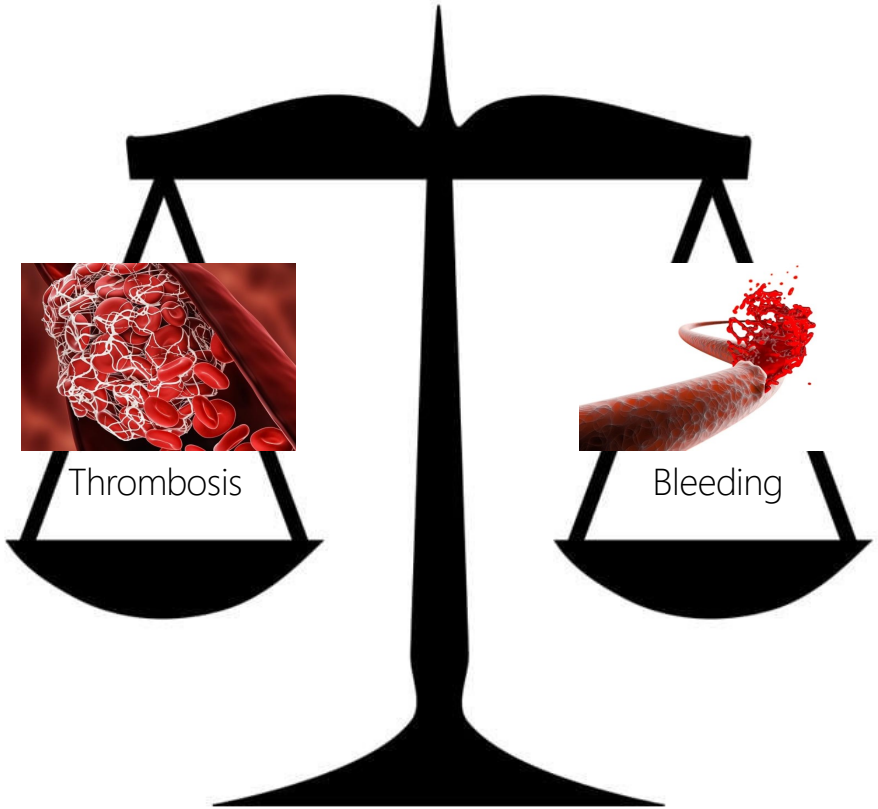


Vandenbroucke *et al.*, 2001 N. Engl. J. Med.

Therapeutics influence haemostasis

Activation coagulation ↑

Reduction bleeding ↓



Reduction coagulation ↓

Activation bleeding ↑

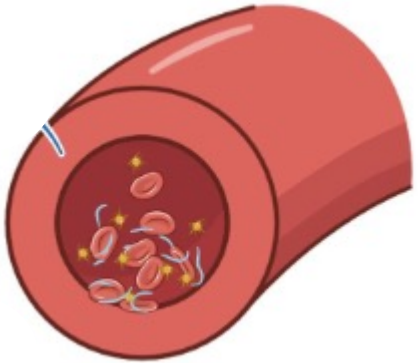
Importance of haemostatic risk assessment for drug approval

- Nearly 90% of drugs fail during clinical trials
- Unacceptable toxicity; ~ 60% (preclinical toxicology and clinical safety).
- Cardiovascular-related complications often cited as primary driver of drug attrition
- Alterations in coagulation/fibrinolysis play a critical role in risks
- Drug safety related to haemostatic risk has long been underestimated and received too little attention

Schultze *et al.*, 2013 Toxicologic Pathology, Waring *et al.*, 2015 Nat. Rev. Drug. Disc.

Haemostasis: Challenges in (pre)clinical sample analysis

- Sample volumes required in preclinical setting
  - No/limited multiplexing possible
- Monitoring endogenous biomarkers
  - Drug interference/ endogenous marker different in human and animal
  - Matrix interference
  - Lot-to-lot variation in kits
- Sample handling requirements
- Complexity of tightly regulated coagulation system

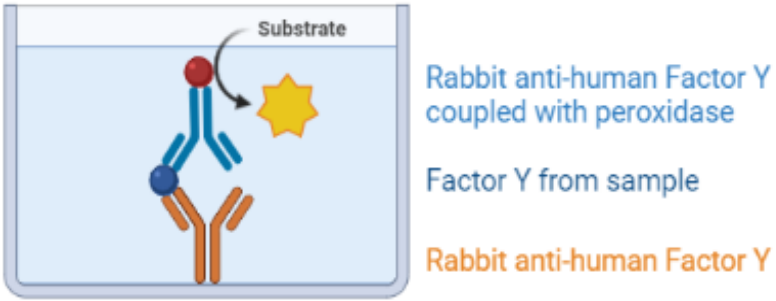


Case studies

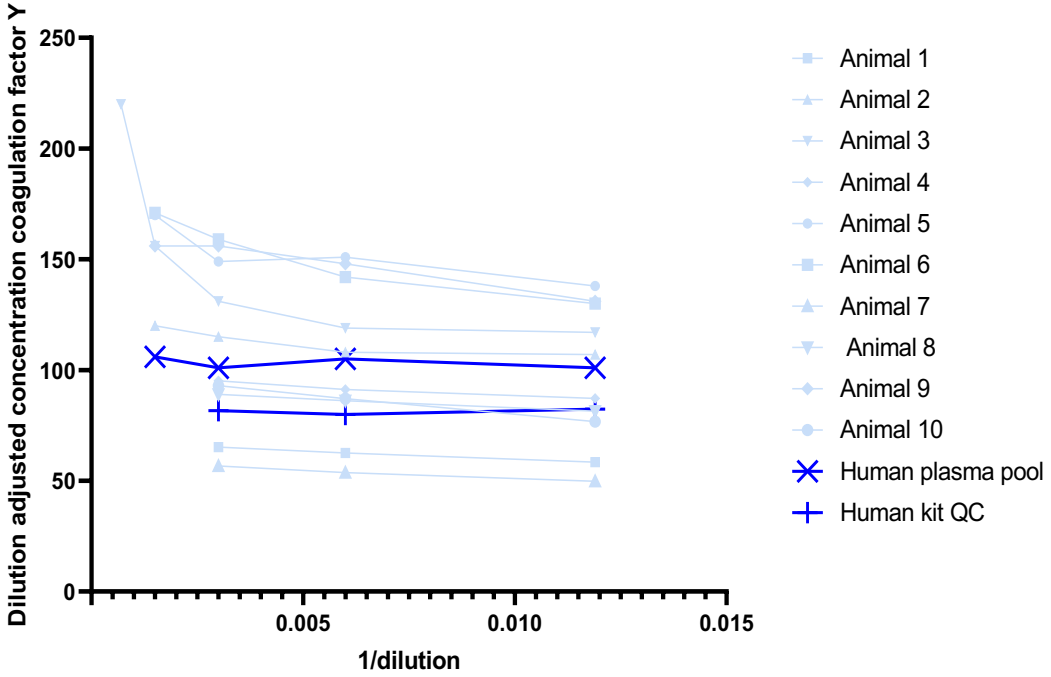
Pre-clinical sample analysis in human ELISA

Coagulation factor Y - Perform Context of Use validation and preparation of QC samples

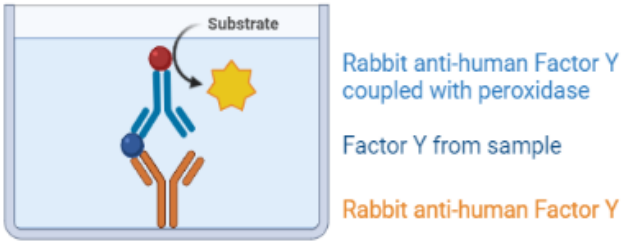
- Ten individual cyno plasma samples measured for endogenous coagulation factor Y.
- Determine QCs for validation (QC-high, QC-mid, QC-low).
- Prepare human spiked recombinant protein in QC-mid
  - when increase in factor Y basal levels is expected.
- Prepare diluted QC-low in human factor Y depleted plasma
  - when decrease in factor Y basal levels is expected.



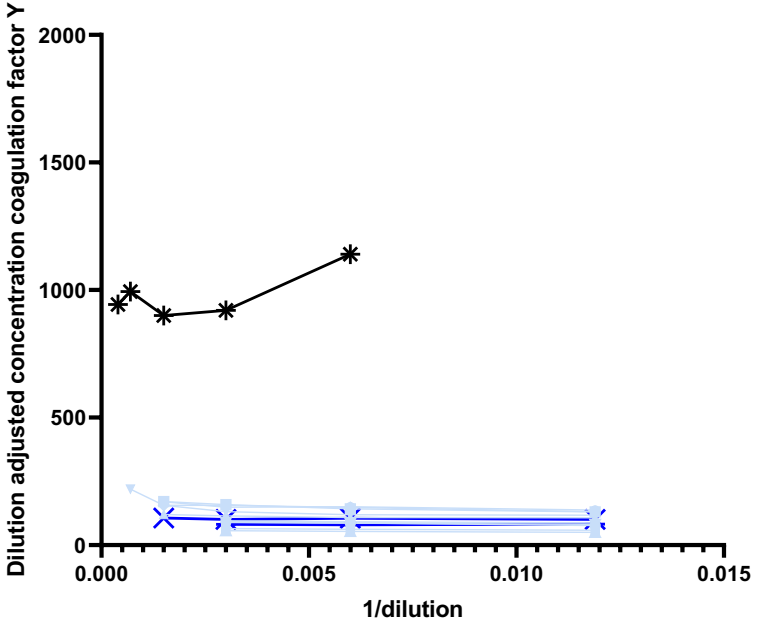
Factor Y ELISA assay parallelism



Sample parallelism proves **selectivity.**

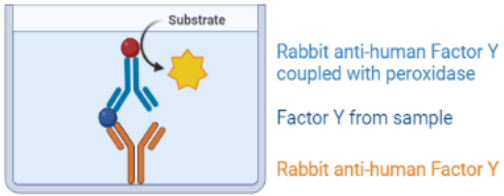


Dilutional linearity of spiked human recombinant factor Y



Dilutional linearity proves **selectivity** and shows proper quantification of **increased concentrations of coagulation factor Y**

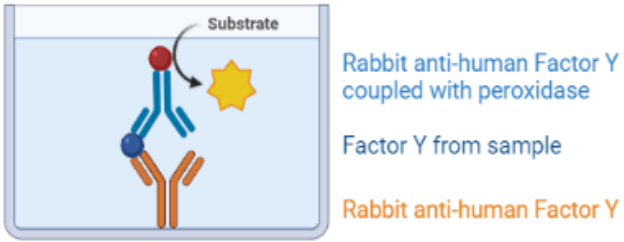
Back-calculated values never differ more than 20%



QC-low shows dilutional linearity in human factor Y depleted plasma

QC samples in depleted plasma	Run 1	Run 2	Run 3	Mean Run 1-3	CV (%)	Recovery (%)
QC-High	112	109	99.4	107	6.0	
QC-Mid	49.1	55.4	50.5	51.6	6.4	
QC-Low	40.3	40.8	N/A	40.5	1.0	**100**
QC Low 50% diluted in depleted plasma	18.6	17.2	20.7	18.8	9.4	93
QC Low 75% diluted in depleted plasma	9.79	10.8	10.8	10.5	5.6	103
QC Low 87.5% diluted in depleted plasma	5.12	7.01	6.64	6.25	16	123

accurately measure low concentrations



Reporting

- Refer to the Context of Use
- Ensure to mention that human proteins/kits/plasma are used for preclinical assay development/validation of an animal protein.
- Refer to the method set-up for human used for cyno as semi-quantitative.
- Report data as human equivalent (*i.e.* nM human equivalent Factor Y).

Sample collection and handling

1. Potential activation of coagulation when performed inaccurately
2. Instability of coagulation proteins (higher stability in 3.2% citrate plasma)

<u>Potential effects:</u>

1. Analysis of individual samples *vs.* pools
2. Venipuncture effects – unwanted activation of coagulation
3. (In)activation of coagulation proteins

aliquot variation in coagulation assay

Each run represents different aliquot
Aliquots taken at same day from same animal show differences

Inter-assay runs		Run 1	Run 2	Run 3	Run 4	Run 5	Run 6			
	Theoretical Activity (mIU/mL)	Activity (mIU/mL)	Activity (mIU/mL)	Activity (mIU/mL)	Activity (mIU/mL)	Activity (mIU/mL)	Activity (mIU/mL)	Mean Activity (mIU/mL)	Accuracy (%)	% CV
QC-low cyno sample	76.0	53.5	123	126	49.2	53.8	105	85.1	112	43
Kit QC 1 LOW	61.0	54.9	65.5	67.4	61.9	64.2	64.7	63.1	103	7
Kit QC 2 HIGH	153	153	163	166	156	153	147	156	102	4

Intra-assay runs		Sample 1	Sample 2	Sample 3			
	Theoretical Activity (mIU/mL)	Activity (mIU/mL)	Activity (mIU/mL)	Activity (mIU/mL)	Mean Activity (mIU/mL)	Accuracy (%)	% CV
QC-low	76.0	124.4	124.7	120.4	**123**	**162**	**2**

Aliquot used for ''QC low value" differed from aliquots taken at same day from same animal show differences

Pooling sample QC aliquots

Use pooled aliquoted QC samples to average out differences in sample storage/shipping/handling before arrival at the test location.

Inter-assay runs		Run 1	Run 2	Run 3	Run 4	Run 5	Run 6			
	Theoretical Activity (mIU/mL)	Activity (mIU/mL)	Activity (mIU/mL)	Activity (mIU/mL)	Activity (mIU/mL)	Activity (mIU/mL)	Activity (mIU/mL)	Mean Activity (mIU/mL)	Accuracy (%)	% CV
QC-low	142.0	153.2	127.6	155.5	137.3	146.3	158.4	**146.4**	**103**	**8**
Kit QC 1 LOW	61.0	69.4	55.8	66.3	63.6	63.6	74.9	**65.6**	**108**	**10**
Kit QC 2 HIGH	153.0	167.9	136.0	154.5	147.9	161.5	175.9	**157.3**	**103**	**9**

Intra-assay runs		Run 1	Run 2	Run 3			
	Theoretical Activity (mIU/mL)	Activity (mIU/mL)	Activity (mIU/mL)	Activity (mIU/mL)	Mean Activity (mIU/mL)	Accuracy (%)	% CV
QC-low	142.0	153.3	154.3	152.1	**153.2**	**108**	**1**

Assay kit lot-to-lot variation

During a pre-clinical sample analysis, lot of coagulation assay kit was changed.

Two challenges were encountered:

1. Bias between lots
2. QCs of new lot were out of spec

Sample name	Result mIU/mL lot A	Result mIU/mL lot B	Bias lot B vs lot A(%)
Control 1 lot A (46 - 76 mIU/mL)	62.8	69.0	10
Control 1 lot B (47 - 79 mIU/mL)	59.4	67.1	13
Sample 1	241	272	13
Sample 2	158	182	15
Sample 3	165	195	18
Sample 4	148	179	21
Sample 5	90.8	101	11
Sample 6	156	190	21
Sample 7	280	330	18
Sample 8	346	416	20
Sample 9	140	173	24
Sample 10	64.8	75.5	17
Control 2 lot A (129 - 177 mIU/mL)	154	187	22
Control 2 lot B (131 - 181 mIU/mL)	160	183	14

Assay kit lot-to-lot variation

Run 1 - Lot A			
Name	**Result (mIU/mL)**	**Target (mIU/mL)**	**Recovery (%)**
Control 1 (lot A)	64.4	61	106
Control 2 (lot A)	172.2	153	113
Calibrator (lot A)	307.9	321	96
Control 1 (lot B)	60.3	63	96
Control 2 (lot B)	174.2	156	112
Calibrator (lot B)	244.4	322	76
Control 1 (lot A)	59.1	61	97
Control 2 (lot A)	162.8	153	106
Control 2 (lot A - as calibrator)	156.8	153	102

Run 2 - Lot B			
Name	**Result (mIU/mL)**	**Target (mIU/mL)**	**Recovery (%)**
Control 1 (lot B)	81.4	63	129
Control 2 (lot B)	212.3	156	136
Calibrator (lot B)	315.5	322	98
Control 1 (lot A)	77.2	61	127
Control 2 (lot A)	186.8	153	122
Calibrator (lot A)	380.5	321	119
Control 1 (lot B)	76.5	63	121
Control 2 (lot B)	198.7	156	127
Control 2 (lot B - as calibrator)	199.9	156	128

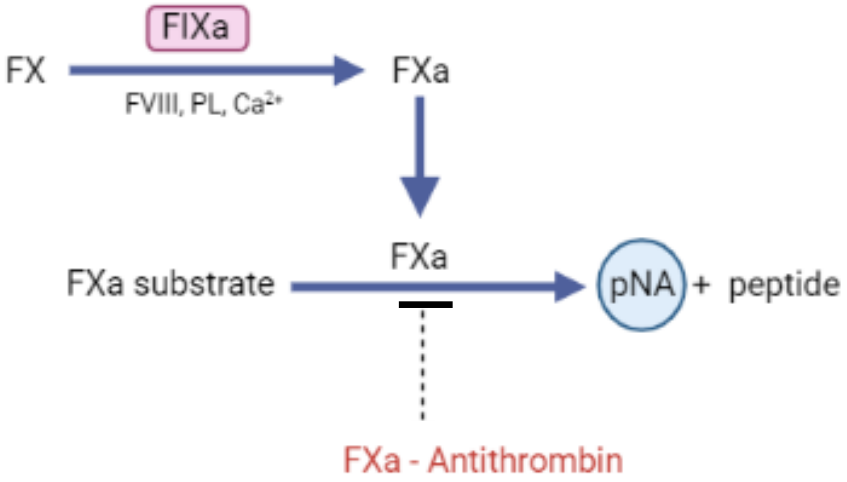
Calibrator of new lot B was too low, resulting in an over-estimation.

Our approach:

Concentration of new calibrator was measured using previous kit lot A and this concentration was then used in preclinical study.

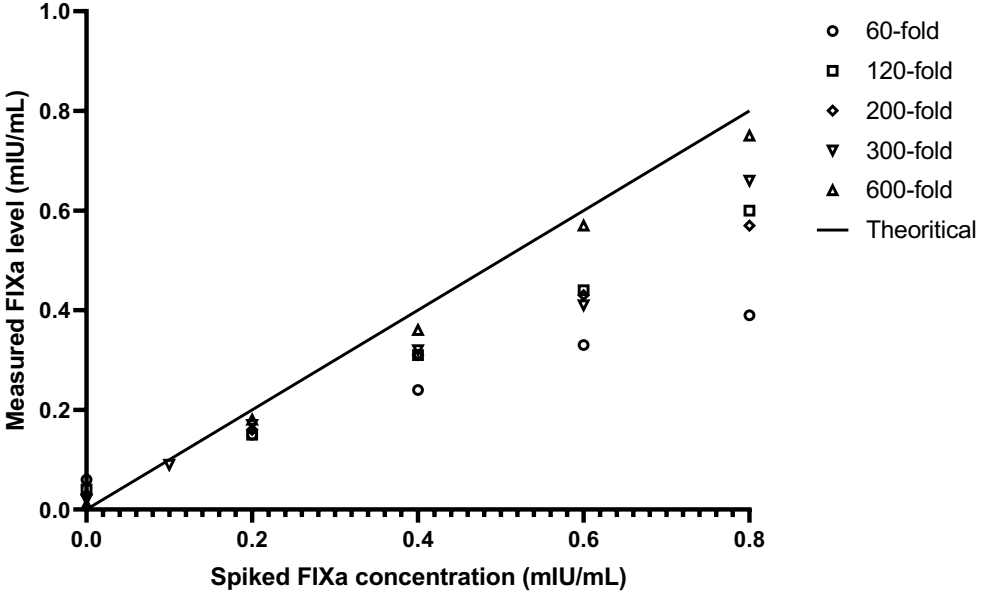
Matrix interference

FIXa activity determination using chromogenic assay



Challenge: Antithrombin present in sample matrix potentially interferes in FIXa activity assay, due to complexation with FXa.

Anti-thrombin interference dilutional effect



- Reduced FIXa levels due to anti-thrombin interference in sample matrix.
- Dilution of matrix in sample buffer decreases matrix effect.
- CoU – Trade off Accuracy *vs.* Sensitivity.

Haemostasis biomarkers are difficult to measure, can be done if you take into account how to

- measure and report endogenous biomarkers to Context of Use principles
- utilise human kits for animal samples
- measure and report (pre)clinical studies
- handle samples
- overcome lot-to-lot variation
- approach matrix/drug interference

Acknowledgements

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