Originating Department	QC
Approval Departments	QC, Validation
Effective Date	Refer to Q-Pulse

1.0 PRODUCT DETAILS

1.1 **Name:** Intrinsic Factor

1.2 **Systematic Name:** Gastric Intrinsic Factor

1.3 **E.C. Number:** 232-711-9

1.4 **Source:** Porcine Stomach Pyloric Section

1.5 **Suitable for BBI Solutions codes:** IF1, IFI1

2.0 ASSAY PRINCIPLE

Intrinsic Factor binds specifically to Vitamin B12. The coating of a Vitamin B12-BSA conjugate to the ELISA plate allows for the extent of Intrinsic Factor binding to be investigated.
A Mouse monoclonal Anti-Intrinsic Factor antibody is used to identify the concentration of Intrinsic Factor bound to the Vitamin B12. The Antibody bound to the Intrinsic Factor on the plate is identified by use of an Anti Mouse AP conjugate. PNPP substrate is used to develop signals, read at an absorbance of 405nm on a plate reader.

3.0 EQUIPMENT REQUIRED

Automatic Pipettes (single and multi-channel).
pH meter (readable to 0.01pH unit).
Analytical balance (readable to 0.1mg).
Heated magnetic stirrer and magnetic followers.
Disposable test tubes, vials and Eppendorf tubes.
Plate Reader.
Stopwatch.
Shaker.
Pipette tips.
Spectrophotometer

4.0 RELATED DOCUMENTS

EOP6769 – Thermo Labsystems Multiskan Ascent 354 Plate Reader
ATMV007 - Assay Method Validation for Intrinsic Factor
QCSR029 – Amendment of the R^2 criterion for the Intrinsic Factor Analytical Procedure

5.0 REQUIRED REAGENT & CONSUMABLE DETAILS

When using the reagents please refer to the manufacturer's instructions for safe handling and disposal.

Reagent	Supplier	Product No.	F.W.
Potassium dihydrogen phosphate (KH_2PO_4) AnalaR	VWR International	26936	136.09
Di-sodium hydrogen phosphate di-hydrate AnalaR	VWR International	28029	177.99
Sodium Chloride	VWR International	27810	58.44
Tween20	Fisher Scientific	10113103	1227.54
Sodium Azide	VWR International	10369AS	65.0
Bovine Serum Albumin (BSA)	Roche	10735108001	
Vitamin B12-BSA Conjugate	CalBioreagents	C093	
Intrinsic Factor Stock for standards	ABD Serotec	5390-0004	
Mouse Monoclonal Anti IF Antibody	Biospacific	A83200415P	
Anti Mouse IgG Whole Antibody AP Conjugate	Sigma Aldrich	A3688	
Eppendorf Tubes (LoBind 1.5ml)	Fisher	10708704	
NUNC Maxisorp plates	Fisher	10547781	
SIGMAFAST pNPP & TRIS BUFFER tablets (pack of 50 pairs)	Sigma Aldrich	N2770-50SET	
SIGMAFAST pNPP tablets	Sigma Aldrich	N2765	Separate alternatives to 50 pack
SIGMAFAST TRIS BUFFER tablets	Sigma Aldrich	T8790	
Rainin LTS Tips (20-300uL)	Anachem	RT-L300 or SR-L300	Purchase most competitively priced

6.0 PREPARATION OF REAGENTS

6.1 Phosphate Buffered Saline (PBS)

Dissolve 1.78g of di-Sodium hydrogen phosphate di-hydrate, 7.2g of Sodium chloride, 0.43g of Potassium di-hydrogen phosphate, and 1.3g of Sodium azide in 800ml of purified water.
Adjust to pH7.2 if necessary with 5.8M HCl or 2M NaOH, and make up to 1 litre with purified water.
Filter the buffer with a 0.2µm filter.
Buffer is stable for 1 month at 2-8ºC once opened, and 6 months at 2-8ºC prior to opening.

6.2 **PBS 1% BSA (PBS 1% BSA)**

Dissolve 10.0g of Bovine serum albumin, along with 1.78g of di-Sodium hydrogen phosphate di-hydrate, 7.2g of Sodium chloride, 0.43g of Potassium di-hydrogen phosphate, and 1.3g Sodium azide in 800ml of purified water.
Adjust to pH7.2 if necessary with 5.8M HCl or 2M NaOH, and make up to 1 litre with purified water.
Filter the buffer with a 0.2µm filter.
Buffer is stable for 1 month at 2-8ºC once opened, and 6 months at 2-8ºC prior to opening.

6.3 **PBS 0.05% Tween20 (Wash Buffer)**

Add 0.5mls of Tween20, along with 1.78g of di-Sodium hydrogen phosphate di-hydrate, 7.2g of Sodium chloride, 0.43g of Potassium di-hydrogen phosphate, and 1.3g Sodium azide in 800ml of purified water.
Adjust to pH7.2 if necessary with 5.8M HCl or 2M NaOH, and make up to 1 litre with purified water.
Filter the buffer with a 0.2µm filter.
Buffer is stable for 1 month at 2-8ºC once opened, and 6 months at 2-8ºC prior to opening.

7.0 TESTING PROCEDURE

7.1 **Coating the plate**

Dilute the B12-BSA Conjugate to 5µg/ml concentration, using **PBS** to dilute.

Note: Make up 10000µl (10ml) minimum per plate for enough volume.

Calculation

5 µg/ml (Conc. required) / Stock µg/ml (Conc. provided)	x	10000	=	Volume (in µl) of stock B12-BSA conjugate to be used (per 10ml)

Dispense 100µl of the B12 conjugate into each well of the plate, cover and incubate on shaker for 1 hour at room temperature, or overnight at 2-8ºC.
Discard the B12 conjugate once the time period has elapsed and wash the plate.

7.2 **Washing the plate**

Dispense 200µl of **Wash Buffer** into each well on the plate, discarding the entire contents of the plate once all the wells have been filled.
Repeat this process 3 times.

Note: At the end of the third wash tamp the plate on to a fresh disposable paper towel to make sure that there is no excess liquid, or air bubbles left in the wells.

7.3 **Blocking of the plate**

Dispense 200µl of **PBS 1% BSA** into each of the wells being used.
Incubate on shaker for at least 30 minutes at room temperature.

Discard the blocking agent once the time period has elapsed and wash the plate (as 7.2).

7.4 Sample dilution and incubation

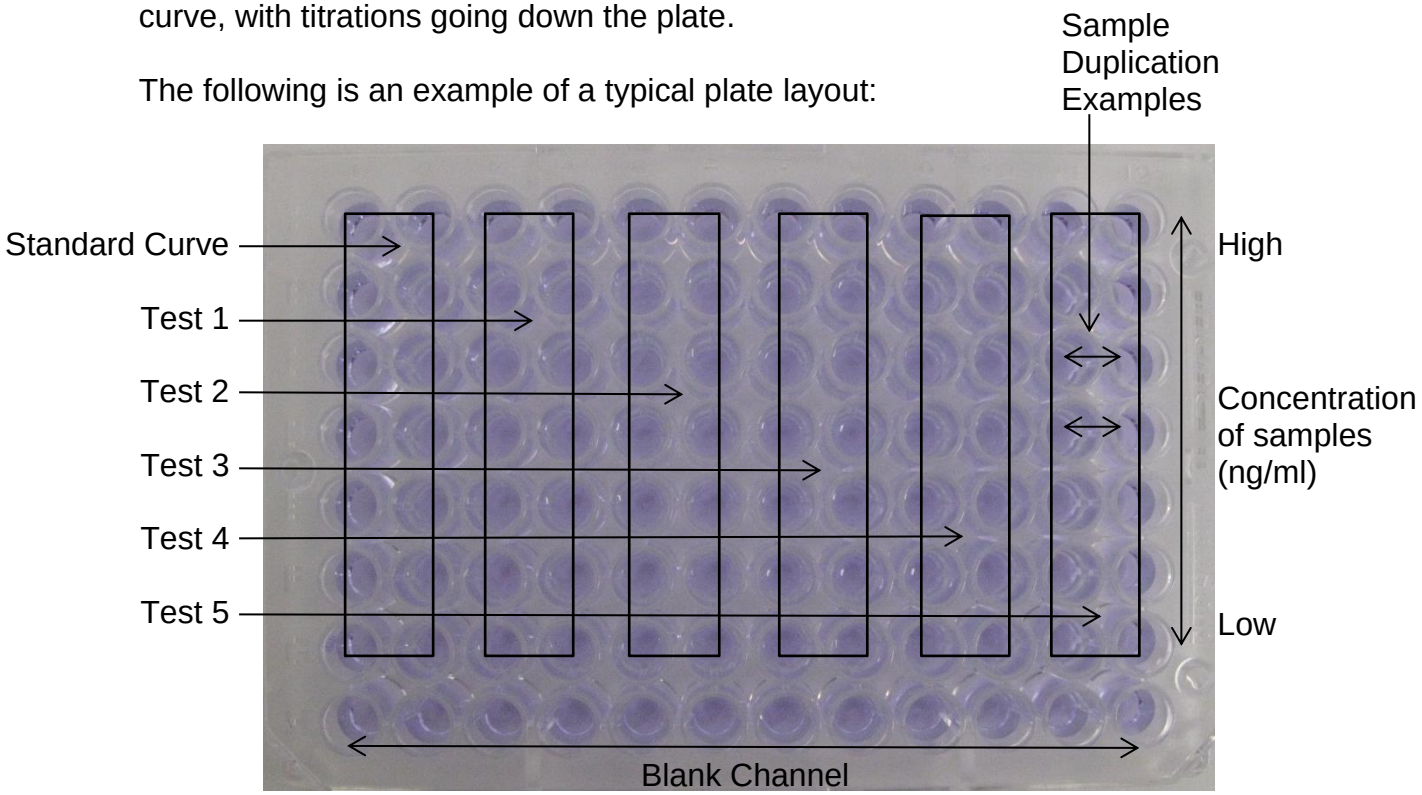
Thaw aliquots of the standards (12.50ng/ml – 0.781ng/ml) at room temperature.

Prepare up to seven (as appropriate) different dilutions for each sample using **PBS 1% BSA** so that the concentrations of the diluted samples will have ng/ml values that plot within the confines of the standards.
The aim is to have as many results as possible within the standard curve. Any results that lie outside have to be discarded.

Note: If, for a given sample, a large number of the dilutions give ng/ml concentrations that are outside the standard curve concentrations the sample will have to be repeated.

Record the position and dilution factor of each sample loaded on to the plate using Appendix 1. Load 100µl of the appropriate sample into each well of the plate, remembering this must be done in duplicate for all samples including the standard curve, with titrations going down the plate.

The following is an example of a typical plate layout:



Once samples have been loaded onto the plate, cover and incubate on the shaker for 1 hour at room temperature.
Discard the stock, diluted and standard curve samples once the time period has elapsed and wash the plate (as 7.2).

7.5 Dilution and loading of the Antibody

Dilute the Monoclonal Anti-IF Antibody to 2µg/ml concentration, using **PBS** to dilute.
Note: Make up 11ml minimum per plate for enough volume.

Calculation

2 µg/ml (Conc. required) / Stock µg/ml (Conc. provided)	x 11000	=	Volume (in µl) of stock Monoclonal Anti-IF Antibody to be used (per 11ml)

Dispense 100µl of the antibody into each well of the plate, cover and incubate on shaker for 1 hour at room temperature.
Discard the antibody once the time period has elapsed and wash the plate (as 7.2).

7.6 Dilution and incubation with the AP conjugate

Dilute the Anti-Mouse IgG AP conjugate to 2.5µg/ml concentration, using **PBS 1% BSA** to dilute.
Note: Make up 11ml minimum per plate for enough volume.

Calculation

2.5 µg/ml (Conc. required) / Stock µg/ml (Conc. provided)	x 11000	=	Volume (in µl) of stock Monoclonal Anti-IF Antibody to be used (per 11ml)

Dispense 100µl of the AP conjugate into each well, cover and incubate on shaker for 30 minutes at room temperature.

Note: While the AP conjugate is incubating the pNPP substrate needs to be made to allow time for the tablets to dissolve. See the following point for preparation instructions.

Discard the AP conjugate once the time period has elapsed and wash the plate (as 7.2).

7.7 Preparation of the pNPP substrate and incubation

The following preparation can cover two plates (if needed). However this substrate should only be prepared when required for immediate use, as the pNPP will naturally colour over time. Any excess should be disposed of after all the wells have been filled to avoid mistaken use at a later date.
Into a vial containing 20ml of purified water place:
1 x SIGMAFAST pNPP tablet and
1 x SIGMAFAST TRIS BUFFER tablet
Using a Vortexer or Rotary Shaker, mix for approximately 25-30 minutes to ensure both tablets fully dissolve.
Filter the substrate with a 0.2µm filter prior to application on the plate.

Dispense 100µl of the pNPP substrate into each well, cover and incubate on shaker for 20 minutes at room temperature.
Use a stopwatch to time this section of the procedure.

7.8 Reading of the ELISA plate

Once **20 minutes** of the incubation of pNPP substrate has elapsed read the absorbance on the plate reader at 405nm.

Note: The plate needs to be inserted into the plate reader and analysis started **within 30 seconds of the twenty minute incubation** time as the colour reaction with the pNPP substrate will continue to take place.

8.0 DATA ANALYSIS

Note: Before calculations are started copy and paste the raw data on to the 'Raw Data' excel work sheet.

8.1 Average Background Reading (Average Blank)

Blank values wil be averaged (usually row H). This average value will be used in all the following calculations when the term 'Average Blank' is used.

Note: The Average Blank for each plate will differ slightly so each value is plate specific.

8.2 Absorbance Values for Use in Standard Curve and Sample Calculations

This calculation applies to all values including the standard curve.

$\frac{\text{Test Rep 1 + Test Rep 2}}{2}$	–	Average Blank	=	Standard/Sample Absorbance Value (Ave Abs – Ave Blank)

8.3 Producing the Standard Curve

The y axis plots the standard absorbance values, against the corresponding concentration values (in ng/ml) on the x axis. The absorbance values can be directly inserted into the excel graph on the 'Standard Curve' worksheet.
The graph should produce a linear curve equation for use in calculating the extrapolated activity for each test sample. The graph should also yield a linear relationship with an R^2 value greater than or equal to 0.98. If this is not the case then this particular plate has failed and the sample dilutions on it should be repeated.
Record the constants generated from the standard curve equation in the appropriate cell on the 'Standard Curve' worksheet ready for use in the sample calculations.

Note: The standard curve will differ slightly for each plate so each curve is plate specific.

8.4 Calculation of Extrapolated Activity

The equation generated for the standard curve is laid out as follows:

$$y = (\textbf{Constant 1})x\ (\pm\textbf{Constant 2})$$

The constants are recorded separately by the analyst on the 'Standard Curve' worksheet to be used in the following rearranged calculation:

$$x = \frac{y\ (\pm\textbf{Constant 2})}{(\textbf{Constant 1})}$$ (Where: y = Sample Absorbance Value)

The rearranged equation can now be used to work out the extrapolated concentration values (x).

Note: The activity calculations following this are only carried out if the extrapolated ng/ml values lie within the boundaries of the graph.
More specifically, the value has to be (less than) <12.500 ng/ml and (greater than) >0.781 ng/ml for the result to be deemed reliable.
If the results obey these criteria, they may be worked through with any other results to completion.

Volume Activity (U/ml) = Extrapolated Conc. x Dilution factor

$$\text{Weight activity (U/mg material)} = \frac{\text{U/ml}}{\text{Mg material/ml}}$$

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8.5 A_{280} Readings

Read using a spectrophotometer, the absorbance at 280nm is a measure of the protein concentration of in-process and final product samples.
Depending on the sample tested, an appropriate dilution buffer must be used to zero the spectrophotometer and dilute the sample (if required) as directed by point 10.0 of this analytical procedure.

8.6 Protein Concentration

$$\text{Protein concentration (mg protein/ml)} = \frac{A_{280}}{1.4}$$

9.0 STANDARD CURVE PREPARATION

9.1 The standard curve comprises of seven standards.

These should be made up from an externally sourced batch of IF (the supplier and product number have been stipulated in point 5.0 of this document).

E.g. For an incoming material with an activity of 0.5 mg/ml (so giving an activity of 500000 ng/ml) the stock was diluted down to 100 ng/ml and from there the individual standards were diluted.

These stocks could then be sub packed, labelled and frozen to allow repeated use at a later date.

Standard Number	Concentration of Standard (ng/ml)
7	12.500
6	9.375
5	6.250
4	4.688
3	3.125
2	1.563
1	0.781

10.0 In-process and Final Product Sample Testing Lists

IFI1 (Intermediate Intrinsic Factor)

Sample	Concentration (ng/ml)	A_{280}	Ng/A_{280}	Total A_{280}	Buffer Used for A_{280}
Clarified 25%	✓				N/A
DES Column Fractions (1-8)	✓	✓	✓	✓	PBS
Conc & Diaf		✓			HQ Water
Final Product	✓	✓	✓		HQ Water

IF1 (Purified Intrinsic Factor)

Sample	Concentration (ng/ml)	A_{280}	Ng/A_{280}	Total A_{280}	Buffer Used for A_{280}
B12 Column Fractions (F2 3.75M & F3 5.625M)	✓	✓	✓	✓	HQ Water
Ex PS U/F		✓			HQ Water
Comb Ex SEC Column Fractions		✓			50mM KPO_4/150mM $NaCl_2$, pH7.5
Ex SEC U/F		✓			HQ Water
0.2µm Filt		✓			HQ Water
Final Product	✓	✓	✓	✓	HQ Water

11.0 REVISION HISTORY

Document Issue Number	Section Number	Summary of Changes
03	N/A	Originating/Approvals section updated to current practice of Effective Date being Refer to Q-Pulse
	4.0	ATMV007 and QCSR029 added to related documents
	8.3	R^2 criterion for standard curve amended to greater than or equal to 0.98 (Amendment supported by QC Study Report QCSR029)