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STABILIZATION OF RM1AND RM2 HUMANIZED MONOCLONAL ANTIBODIES (h~mAb's) LIQUID FORMULATION USING A SURFACTANT

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ABSTRACT

Advent of genetically engineered humanized monoclonal antibodies (h~mAb's) are accelerated the need of its formulation in oncology therapy and emerging as the fastest growing area of Biopharmaceutical Formulations. Being the (h~mAb's) is a protein with high degree of specificity and selective target recognition in nature, its formulation poses the major technical challenge on stability of the antibodies in various configurations particularly when it is designed for systemic administration typically at larger concentrations. This research focuses on the role of surfactant as a stabilizing agent in the formulation of two humanized antibody based drugs. These antibodies stability was established by using a validated Size Exclusion Chromatography, and SDS-PAGE method. In order to achieve sufficient stability of antibody-based material a market survey of the existing similar formulations was studied, evaluated, gained the knowledge of stabilizing agent in formulation and a non ionic surfactant Tween 80 (Polysorbate 80) was selected to stabilize the formulation at various concentrations ranging from 0.01% to 0.5% in phosphate buffer pH 7.2 . The results indicated that RM1h~mAb and RM2 h~mAb was stable at under the shaking conditions and in extended storage at 250C. Multiple freeze / thaw cycles of RM1 increased the level of antibody aggregation and this seemed to be increased by the presence of Tween 80 in the formulation buffer. Whereas Multiple freeze / thaw cycles of RM2 showed an apparent increase in the level of antibody aggregation, although the levels were below 3%. This increase is independent of the level of Tween 80 in the formulation buffer.

Keywords *– Monoclonal Antibodies, Surfactant, Natural gums, Antibody, Biopharmaceutical Formulation.*

1. INTRODUCTION

Over hundreds of monoclonal antibodies are in clinical trials and more than 20 mAb‟s and two bio-similars are currently in the market as approved mAb‟s. With the advances in the genetic engineering in the pharmaceutical filed, wreak a new emerging field as Biopharmaceutical Formulation Sciences and also bring into being as fastest growing class of human pharmaceuticals. Relatively Humanized Monoclonal Antibody (protein) drugs has for long been a problem in formulation of a suitable pharmaceutical dosage forms since these are being protein in nature and complex limits the aspects such as appropriate drug delivery system and Stability. In this research two Humanized Monoclonal Antibodies (h~mAb‟s) are used in the formulation

development that are at the stage of phase-I clinical trial development. The first one is "RM1- h~mAb" is indicated therapeutically for the treatment of Lung cancer and the second one is "RM2 h~mAb" is indicated for Breast Cancer. These two antibody based drugs contain human IgG1 class constant regions and are glycosylated on the Fc (fragment crystallizable) domain. Glycosylation has been shown to participate in specific cell response events mediated by antibodies such as antibody- dependent cell mediated cytotoxicity (ADDC) and complement- dependent cell mediated cytotoxicity (CDCC) through receptor recognition events.

In vitro and in vivo studies of these two antibodies (RM1 and RM2) have proven a drug candidate that induces target tumor cell killing through complement-dependent CDCC and ADDC. The physicochemical structure of recombinant humanized (RM1 and RM2) are similar to that of circulating IgGs existing in >10 g/L concentration in human serum. By themselves, these molecules are potentially less toxic than xenobiotic small drugs.

These mAb‟s due to protein in nature, present formulation issues like degradation under conditions when they are exposed to heat, freezing, light, pH extremes, Agitation, shear stress, some metals and organic solvents. In addition to these issues is the difficulty of preparing dosing materials that have protein concentrations in the range of 10 mg/mL or greater. The present invention relates to a formulation effort was made to employ " isotonic, non-hemolytic, slightly alkaline solution that can be administered by intravenous injection with appropriate stabilizing agent, Polysorbate 80 (Tween-80) and a suitable buffering agent against exogenous stress.

On the evaluation of the existing available marketed h~mAb‟s formulations as shown in Table-1 and keeping the better targeted product profile thus the preparation of Intra Venous solutions (Parenteral dosage form) would be advantageous to study the proof of concept of h~mAb1 and h~mAb2 as a phase-I studies in humans for first time. This also it is eased and convenience of administration coupled with faster therapeutic action.

Novel concern related to the formulation of h~mAb‟s are its delivery strategies for such large amounts of a complex molecule and its formulation development in to a liquid form of intravenous stable formulations with regard to physicochemical and thermodynamic events becomes more and more critical to ensure market success. In order to overcome these problems different concentration of Tween 80 was used and optimized to maximize the physical and chemical stability thus retaining its biological / therapeutic activity on the cancer. In this research an attempt was made to optimize the required concentration of Tween 80 for better stability or protection of these h~mAb‟s from its degradation/aggregation upon Shear stress, Shaking, Freeze/Thaw cycling and from the temperature on shelf life.

In the pharmaceutical industries Tween 80 is have been largely used in protein solution formulations to solubilized and to stabilize the protein. Tween 80 (also known as Polysorbate 80) is a non ionic surfactant and is polyoxyethylene 20 sorbitan monooleate.

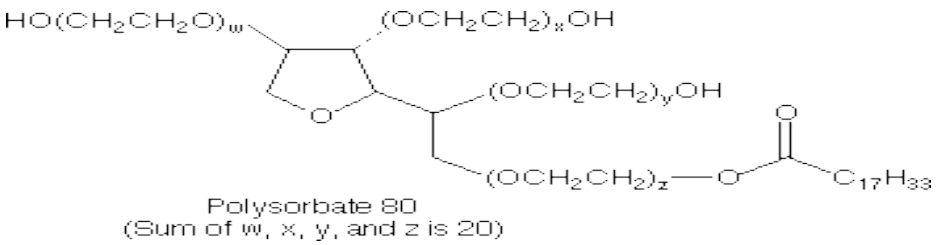


Fig. 1: TWEEN 80 Structure

Some of the literatures are listed here in respect to the protection of proteins by Tween 80. Low concentrations of nonionic surfactants are often sufficient to prevent or reduce protein surface adsorption and or aggregation due to their relatively low critical micelle concentrations (CMC) (Bam et al. 1995 and Nema and Avis, 1993; Carpenter et al., 1997). Complete or significant

inhibition has been reported of surface adsorption loss of TGF-b1 by Tween 80 at0.01% (Gombotz et al., 1996), shaking-induced hemoglobin aggregation by Tween 80 at 0.045% (Kerwin et al., 1998).

Table-1 : List of products approved in the market and its formulation details

mAb – Drug product	*Company Name*	*Generic Name and description*	*Delivery Route*	*Dosage and Strength*	*Formulation details(excipients) stabilizing agents, buffers and pH*
Humira® (Adalimumab)	Abbott	Adalimumab; Humanized IgG1,ant-TNFα; Anti inflammatory, auto immune disorder	IV	50mg/ml	Mannitol,Na phosphate, Na citrate, Polysorbate 80, pH 5.2
Tysabri (Natalizumab)	Biogen idec	Natalizumab; humanized,IgG4 , anti-4α-integrin; autoimmune-multiple sclerosis therapy	IV	20mg/ml	Na phosphate, NaCl , polysorbate 80, pH 6.1
Campath® - IH (Almetuzumab)	Millennium -ILEX	Almetuzumab; Humanized IgG1, anti-CD52 ; Chronic lymphocytic leukemia	IV	30mg/ml	Na,K phosphate, NaCl, KCl, Polysorbate 80, pH 7.0
Zenapax® (Daclizimab)	Roche	Daclizimab; Humanized IgG1; anti-CD25	IV (lyophilized)	5mg/ml	Na Phosphate, NaCl, Polysorbate 80, pH 6.9
Avasatin® (Bevaclzumab)	Genetcech	Bevaclzumab; humanized IgG1,ant-VEGF; colorectal cancer	IV	25mg/ml	Na phosphate,Trehalose, Polysorbate 20, pH6.4

There are several possible mechanisms suggested in the literature for protein protection by surfactants. One mechanism is specific binding/ interaction with hydrophobic sites on the protein, where Tween 80 can bind weakly to the protein surface (Bam et al., 1995; Katakam etal., 1995; Bam et al., 1998). The protective ability may then correlate with the amount of surfactant needed to saturate these hydrophobic patches (the molar ratio of surfactant to protein).

The toxicology batches of RM1 and RM2 were completed and were formulated into phosphate buffer saline (PBS) pH 7.2 at a concentration of 10 mg/mL based on A280nm. This material has been stored at 2-8°C since the final formulation.

The following document describes the evaluation of four different formulation (variable with different concentrations of Tween 80) on product quality while subjected to different stresses. These stresses included shaking, multiple freeze thaw cycles and accelerated stability by incubation at 25°C and at 40°C.

The four formulations were as follows

1)10mg/mL in PBS pH 7.2

2)10mg/mL in PBS pH 7.2 + 0.01%w/v Tween 80

3)10mg/mL in PBS pH 7.2 + 0.05%w/v Tween 80

4)10mg/mL in PBS pH 7.2 + 0.1%w/v Tween 80

These formulations were created by the addition of a concentrated Tween 80 solution (5%w/v) to the bulk drug substance in a way that minimized the dilution of the product.

The various conditions and stresses were analyzed as single samples, with exception of the elevated temperature stability, where duplicates sample was needed for analysis.[1-13]

2.MATERIALS AND METHODS

The materials used in the study are summarized in the table-2.

Table 2: List of materials used in the formulation and testing of the RM1 and RM1

Name of the Material Equipment /Instrument used	Make	Supported by
RM1 and RM2 Humanized monoclonal Antibodies stored at -20 °C and 2-8°C	Shantha Biotechnics	Shantha Biotechnics
Sodium Phosphate Buffer Saline Solution (PBS) pH 7.2 $Na_2HPO_4*2H_20$ (MW = 178.05)	Sigma Aldrich	Shantha Biotechnics
Sodium Chloride [(150mM NaCl) M.W= 58]	Merck	Shantha Biotechnics
Tween 80 (Polysorbate 20 monoleate)		Shantha Biotechnics
Water for injection	In House Christ aqua.	Shantha Biotechnics
Ammonium persulfate (APS) ($N_2H_8S_2O_8$; mW: 228.2)	Merck	Shantha Biotechnics
HPLC	Waters Liquid Chromatography	Shantha Biotechnics
Column Used: TSK 3000 SWxL (30 x 7.8 cm)	Tosho Bioscience	Shantha Biotechnics
Sterilized Glass Beaker, Measuring Cylinders, micro- pippets, Sterilized USP Type-I Vials, Mechanical Stirrer,	Thermolab /electrolab Thermofischer scientific	Shantha Biotechnics
Reverse Laminar Air flow (Class 100)/Bio safety cabinet, Autoclave , Syringes.	Thermofischer	Shantha Biotechnics
Stability chambers with 2-8ºC, 25ºC 60±5%RH, 40ºC70±5%RH.	Rotronic	Shantha Biotechnics
Gel Electrophoresis (SDS- PAGE)	BioRad	Shantha Biotechnics

2.1 Formulation Experimental Work and Procedure Methods

2.1.1 Preparation of samples

Reagents

(1)PBS – this was the current formulation (10mM sodium phosphate + 150mM NaCl pH 7.2)

(2)PBS + 5% w/v Tween 80 NF, the above PBS formulation with the addition of 5g of Tween 80/L.

2.2 Formulation of Product

440mL of RM1 andRM2 h~mAb‟s was removed from the bulk drug substance and aliquoted into 4 x 110mL containers for each antibody. These were formulated as shown in table-3.

Table-3: Formulation details of the RM1 and Rm2 humanized antibodies

Component	***Formulation -1***	***Formulation - 2***	***Formulation - 3***
Strength	PBS + 0.01% Tween 80	PBS + 0.05% Tween 80	PBS + 0.1% Tween 80
RM1 /RM2 antibody	110 mL	110mL	110 mL
PBS	1.98 mL	1.1 mL	-
5% Tween 80	0.22 mL	1.1mL	2.2 mL

Each adjusted formulation was filtered through a 0.2µm Nalgene filter and aseptically transferred into the number of vials described below. These vials were closed and were then ready for the various studies described below.

2.2.1 Description of the Process

Previous work in qualifying the SEC-HPLC method with RM1 and RM2 antibodies indicated that the linearity of the method is lost below <3% aggregate and as such changes in aggregate levels at 3% or less are not deemed significant.

2.3 Stability Testing Procedures

2.3.1 Shaking analysis

3 x 8ml aliquots were vialed in 10cc vials, per condition per antibody. The samples were shaken at 50 rpm for 24 and 48 hours on an orbital shaker and were then stored at 2-8 ºC prior to analysis. Total number of samples for analysis was 12 x 2 antibodies (single sample per point).

2.3.2 Freeze Thaw

5 x 3ml aliquots were vialed in 10cc vials, per condition per antibody. These samples were frozen at –80 ºC and thawed at 25 ºC. The samples were stored at –80 ºC prior to analysis so that the last thaw will be added to the number of thaw cycles. 1mL of the final sample was shipped at 2-8 ºC to for Activity analysis. Total number of samples for analysis was 20 x 2.

2.3.3 Temperature 25 ºC and 40 ºC Stability

10 x 3ml aliquots were vialed in 5cc vials for RM1 and 10cc vials for RM2, per condition per temperature, per antibody .The samples were incubated at 25 ºC and 40 ºC. A 1mL aliquot was taken at each time point and this, together with the remaining samples, was stored at – (minus) 80 ºC prior to analysis.

2.3.4 Analysis Methods

The following methods were used to evaluate the stability of the above mAb‟s formulations: Size exclusion HPLC (SEC-HPLC) Reducing and non-reducing polyacrylamide gel electrophoresis (SDS-PAGE).

2.3.4.1 SEC-HPLC Analysis for Purity and Insoluble Aggregate Analysis

2.3.4.2 HPLC Instrumentation Details

HPLC make: Water„s Alliance System, HPLC Pump: Water 2695 separation module; Auto sampler: Water 2996, Detector: Photodiode array detector connected to diode array detector, Type of Analysis: Single gradient Type analysis, Software used: Empower;Column Used: TSK 3000 SWxL (30 x 7.8 cm), Maximum Flow rate: 1.2 mL /minute; Standard flow rate 0.5 to 1.0 mL /minute,Operating pH Range: 2.5 to 7.5.

2.3.4.3 HPLC Method

Eluant A: 10 mM Phosphate Buffered Saline (1xPBS) of pH 7.2, Eluant B and C : Nil;Flow rate:
0.5 ml/minute, Run Time: 45 minutes, Gradient: Single Gradient Phosphate buffered saline (1xPBS), Detection: 280 nm (Photo diode array detector),Calibration: 5 point linear curve 5,
10,100, 150 and200 mg/mL calibration based on peak height typical; Standard: IgG standard 10 mg/mL, GFC Marker: Biorad, Injection: 20 µl of samples, Precision: typical C.V. < 1%, Mobile Phase Composition: Phosphate Buffered Saline as follows; Di Sodium hydrogen phosphate (Molecular Weight: 141.96) 1.08g + Mono sodium Hydrogen Phosphate (Molecular Weight: 156.01) 0.37 g + Sodium Chloride 8.76 g + Water for injection (Milli- Q, H2O) Quantity Sufficient and pH adjusted to 7.2 by using NaOH or HCl.

2.3.4.4 Samples Preparation

Directly use formulated solution 10 mg/mL. The formation of irreversible soluble IgG aggregates was monitored with native size exclusion chromatography (SEC), which was performed on a SWXL 3000 (7.8 × 300 mm) column (Tosoh Bioscience, Germany) and a fully automated High performance liquid chromatography (HPLC) system (Water Alliance). Typically, 10-20 µg of h~mAb protein

were loaded onto the column and were eluted with a mobile phase consisting of 10 mM Phosphate Buffered Saline (pH 7.2) at a flow rate of 0.5 mL/min. Protein elution was monitored at 280 nm using a (Photo diode array detector). All samples were run in triplicate and reported results reflect mean values ± 1 SD. Human IgG concentrations were calculated by extrapolation to a standard curve, which was constructed with bulk IgG1 over the range of 1-50 µg (r2 = 0.99998).The results are tabulated as per the stability samples pulled from different conditions and the chromatograms are as depicted in the figures.

2.3.4.5 Structure Analysis by SDS-PAGE analysis

SDS-PAGE analysis is performed by using Biorad-Protean-II instrument with both the techniques of reducing and Non reducing Gel electrophoresis. The reducing Gel electrophoresis is consists of 5% Stacking Gel and 12% Resolving Gel. In the Non reducing it was used 5% stacking gel and 8% resolving gel. The results are tabulated and reported below.

3.0 RESULTS AND DISCUSSIONS

3.1Shaking stability of different Tween- 80formulations of RM1mAb's using SDS-PAGE (Reducing and Non Reducing):

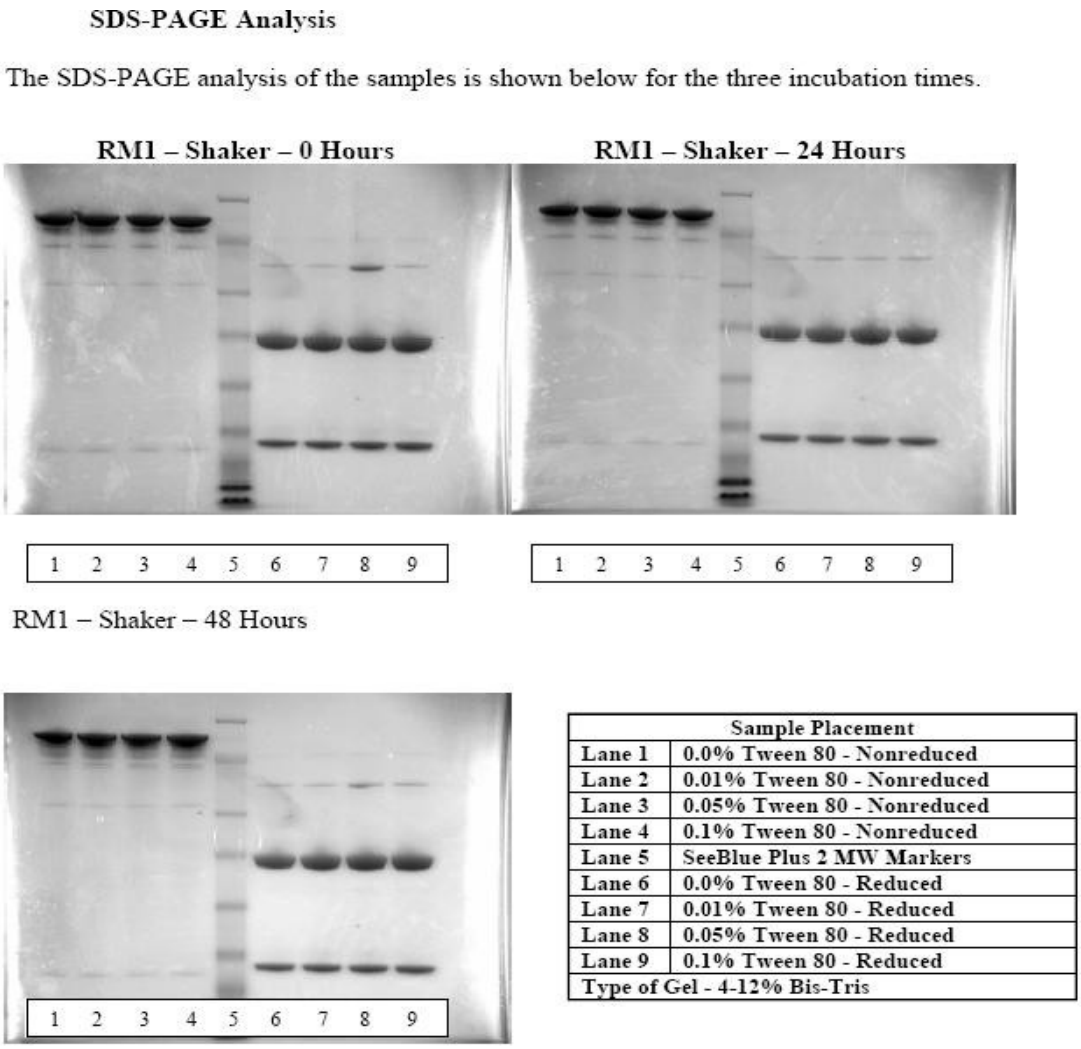
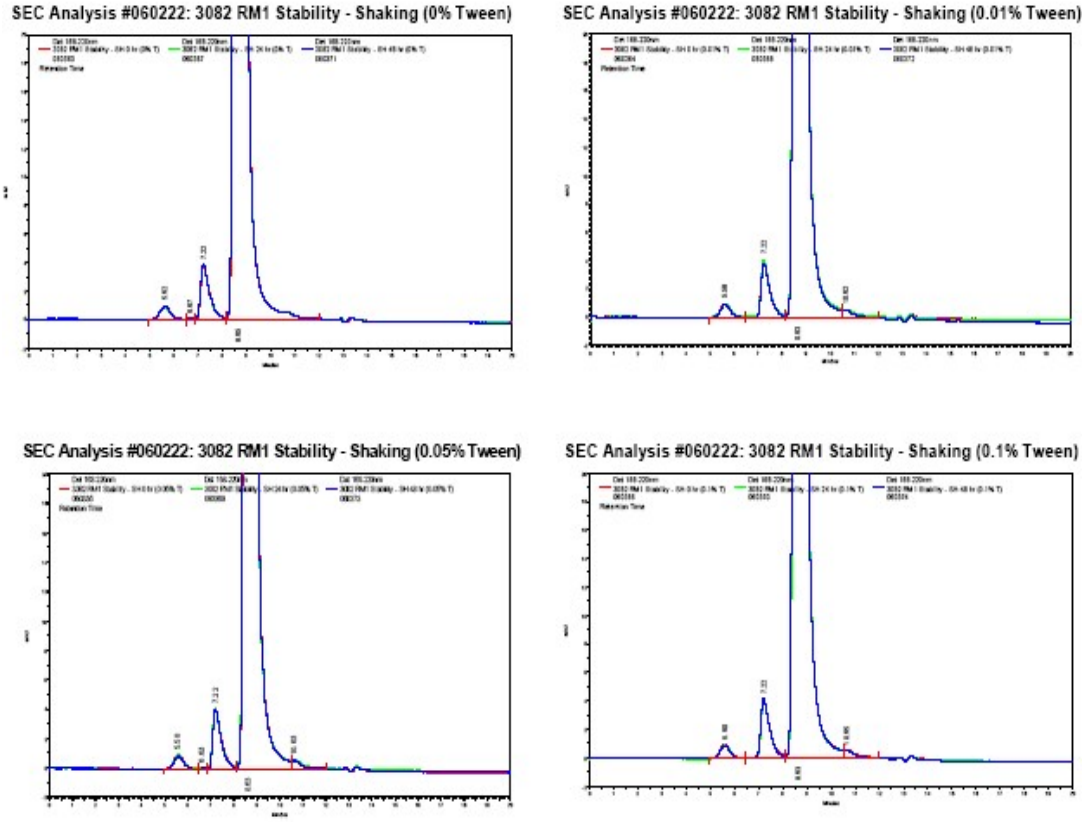


Fig. 2: SDS – PAGE analysis images of RM1humanized mAbs on different Shaking times as part of stability studies for 48 hours on different Tween -80 concentration formulations.

3.3 Shaking Stability of different Tween 80formulations of RM1mAb's using SEC

The SEC-HPLC chromatograms for this study are shown below:



Summary of SEC HPLC Traces

RM1	% Aggregate			
% Tween 80 (w/v)	0.00	0.01	0.05	0.10
0 hr	1.7	1.7	1.7	1.7
24 hr	1.7	1.7	1.7	1.7
48 hr	1.7	1.6	1.7	1.7

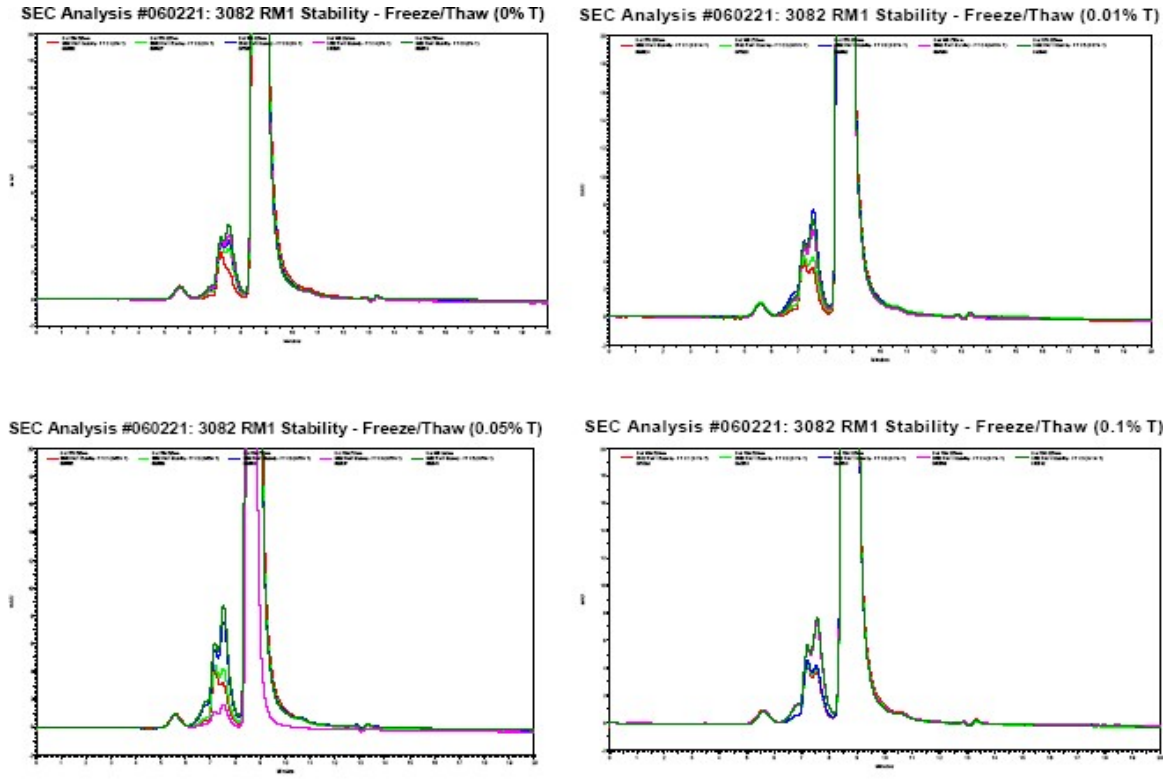
Analysis-SEC 060222

The level of aggregate was not seen to increase during the study and / or across the range of Tween 80 concentrations used in this study.

Fig.3: Chromatograms of SEC analysis of RM1 humanized mAb"s on different Shaking times as part of stability study for 48 hours on different Tween -80 concentration formulations.

3.3 Freeze/Thaw Stability Study of different Tween -80 formulations of RM1mAb's

SEC-HPLC Analysis of Aggregates



Summary of SEC HPLC Traces

The SEC-HPLC chromatograms for this study are shown below:

RM1	% (w/v) Tween 80			
Cycle	0.00	0.01	0.05	0.10
1	1.9	2.5	2.4	2.6
2	2.5	2.9	2.8	2.7
3	2.9	4.4	4.4	2.8
4	3.1	3.7	5.6	4.1
5	3.1	4.0	4.8	4.3

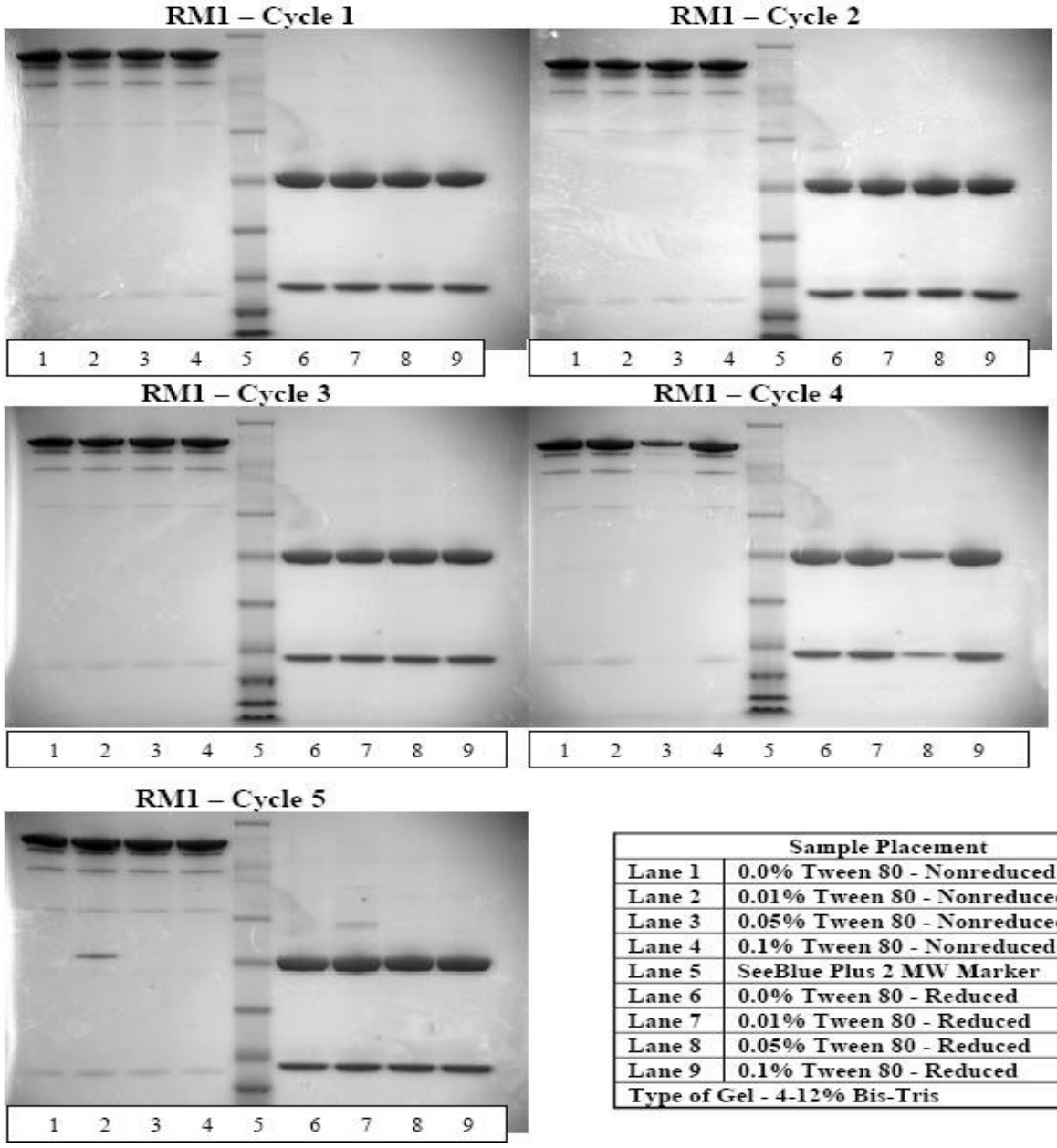
Analysis-SEC 060221

Fig. 4: Chromatograms of SEC analysis of RM1 humanized mAb‟s on different Freeze/Thawing (5 cycle times) as part of stability study on different Tween -80 concentration formulations.

3.4 Freeze/Thaw Stability Study of different Tween- 80formulations of RM1mAb' s by SDS-PAGE Analysis

The SDS-PAGE analysis shows no significant difference occurring to the product during the multiple freeze / thaw cycles and / or across the range of Tween 80 concentrations used in this study. The differences seen in the 0.01% Cycle 5 sample are believed to be an artifact of the gel analysis.

The SDS-PAGE analysis of the samples is shown below for the five freeze /thaw cycles.



Sample Placement	
Lane 1	0.0% Tween 80 - Nonreduced
Lane 2	0.01% Tween 80 - Nonreduced
Lane 3	0.05% Tween 80 - Nonreduced
Lane 4	0.1% Tween 80 - Nonreduced
Lane 5	SeeBlue Plus 2 MW Marker
Lane 6	0.0% Tween 80 - Reduced
Lane 7	0.01% Tween 80 - Reduced
Lane 8	0.05% Tween 80 - Reduced
Lane 9	0.1% Tween 80 - Reduced
Type of Gel - 4-12% Bis-Tris	

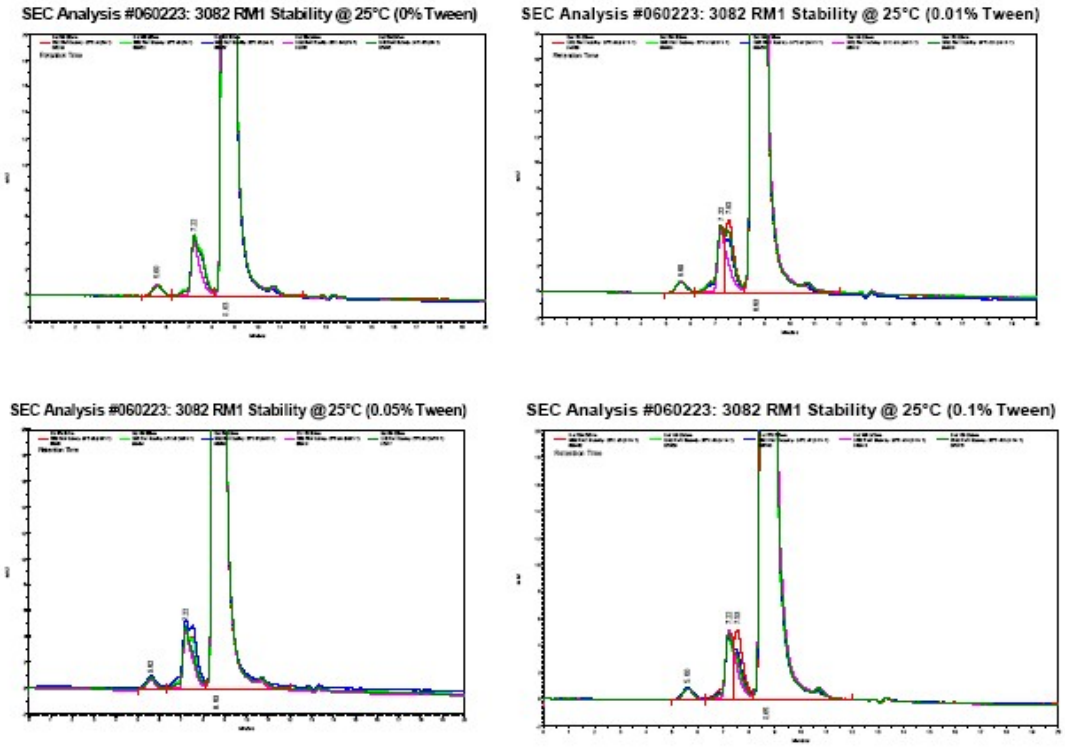
Fig. 5: SDS – PAGE analysis images of RM1humanized mAb‟s of different Freeze thawing cycles (5 cycles) as part of stability study on different Tween -80 concentration formulations.

3.5 Room Temperature Stability of different Tween- 80formulations of RM1mAb's

3.5.1 SEC-HPLC Analysis of Aggregates

The SEC-HPLC chromatograms for this study are shown below:

25°C



Summary of SEC HPLC Traces

RM1	% aggregate			
Temp (°C)	25	25	25	25
% Tween 80 (w/v)	0	0.01	0.05	0.1
0	3.3	2.6	3.1	2.7
3	3.0	2.6	2.2	2.5
7	2.7	3.0	2.5	2.2
14	1.8	1.9	1.7	1.9
21	3.0	2.2	2.4	2.3

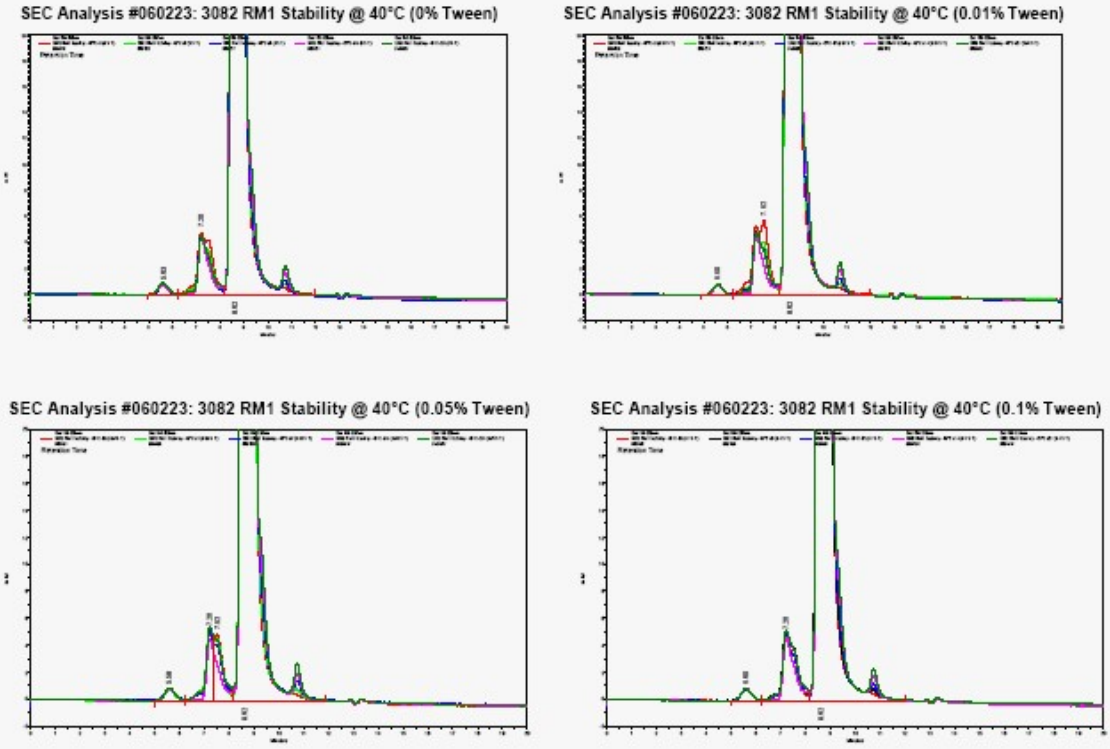
The level of aggregate was not seen to increase during the 21 days of study at 23-28°C and / or not significantly across the range of Tween 80 concentrations used in this study.

Fig. 6: Chromatograms of SEC analysis of RM1 humanized mAb‟s at Room temperature (25°C) stability study on different Tween -80 concentration formulations.

3.5.2 Accelerated Stability of different Tween- 80 formulations of RM1mAb's

The SEC-HPLC Analysis aggregates are shown below

40°C



Summary of SEC HPLC Traces

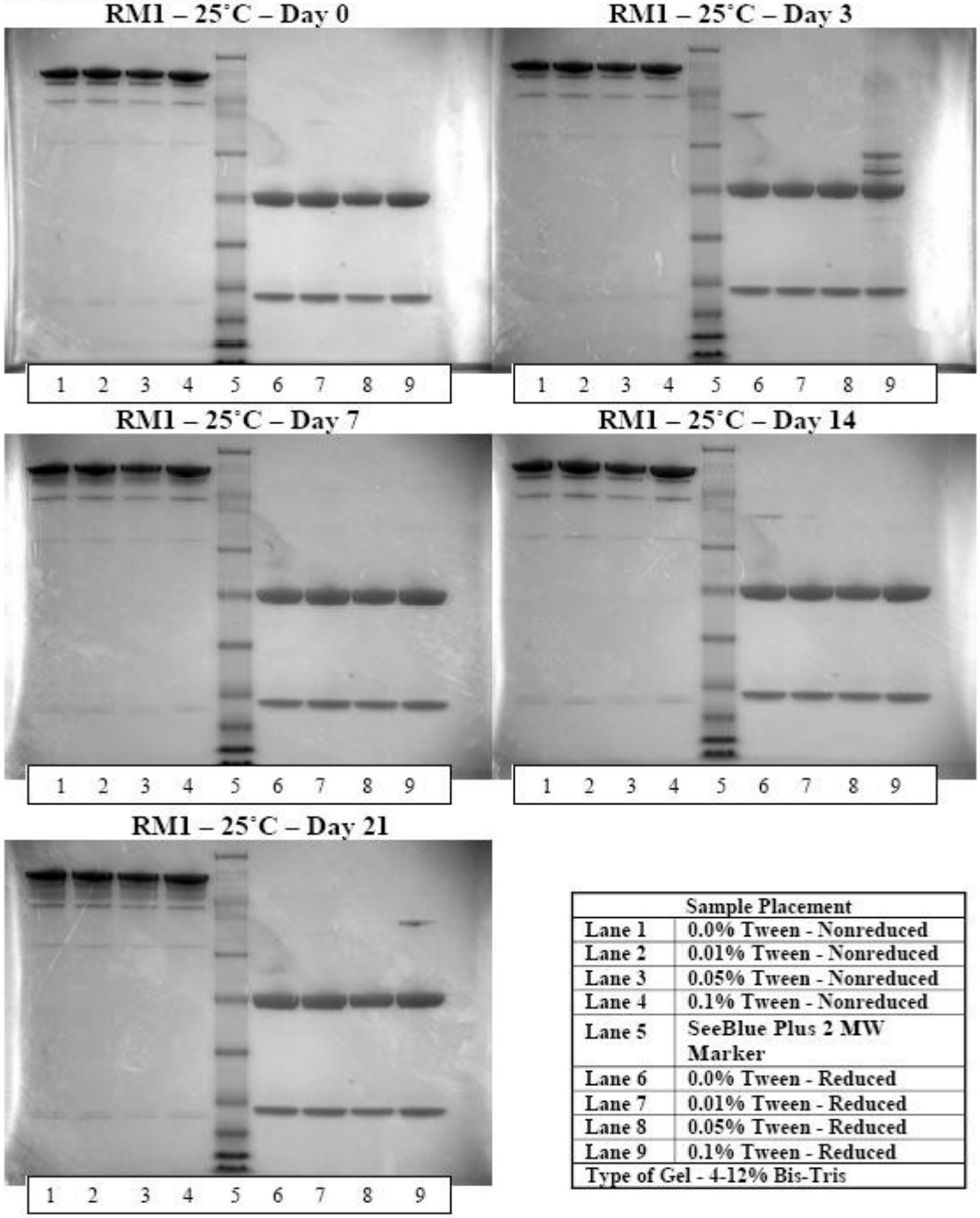
RM1	% aggregate			
Temp (°C)	40	40	40	40
% Tween 80 (w/v)	0	0.01	0.05	0.1
0	3.4	3.0	2.5	3.3
3	2.7	3.0	2.7	3.0
7	2.5	2.7	2.4	2.7
14	2.0	2.1	2.0	1.8
21	2.6	3.0	2.8	3.0

The results do not indicate a significant increase in aggregates over the study period at 40°C. However the level of an apparent degradant does appear to increase and this was observed for all Tween 80 concentrations.

Fig. 7: Chromatograms of SEC analysis of RM1 humanized mAb‟s at accelerated temperature (40°C) stability study on different Tween -80 concentration formulations.

3.5.3 Room Temperature (25°C) Stability of different Tween- 80 formulations of RM1mAb's using SDS-PAGE Analysis for 21 days stability (5 time points)

The SDS-PAGE analysis of the samples is shown below for the five incubation times at the two temperatures.

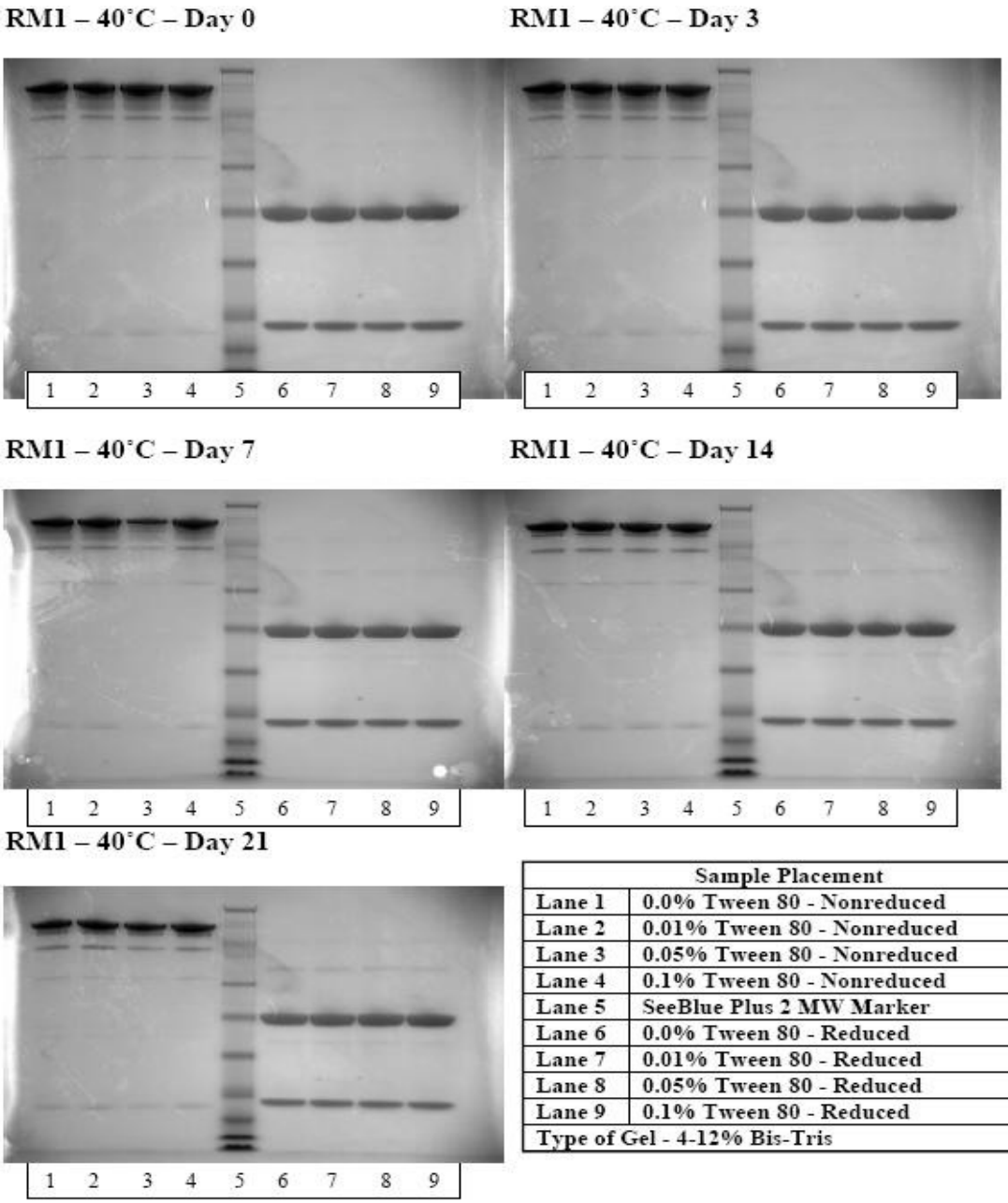


Sample Placement	
Lane 1	0.0% Tween - Nonreduced
Lane 2	0.01% Tween - Nonreduced
Lane 3	0.05% Tween - Nonreduced
Lane 4	0.1% Tween - Nonreduced
Lane 5	SeeBlue Plus 2 MW Marker
Lane 6	0.0% Tween - Reduced
Lane 7	0.01% Tween - Reduced
Lane 8	0.05% Tween - Reduced
Lane 9	0.1% Tween - Reduced
Type of Gel - 4-12% Bis-Tris	

The SDS-PAGE analysis shows no significant difference occurring to the product during the storage at 25°C and there is no apparent difference between the Tween 80 concentrations used in this study. The differences seen in a few of the lanes are not consistent through the study and as such could be attributed to artifacts of the gel analysis.

Fig. 8: SDS – PAGE analysis- images of RM1humanized mAb"s at Room temperature (25°C) stability study on different Tween - 80 concentration formulations.

3.5.4 Accelerated Temperature (40°C) Stability of different Tween- 80 formulations of RM1mAb's using SDS-PAGE Analysis for 21 days stability (5 time points)



Sample Placement	
Lane 1	0.0% Tween 80 - Nonreduced
Lane 2	0.01% Tween 80 - Nonreduced
Lane 3	0.05% Tween 80 - Nonreduced
Lane 4	0.1% Tween 80 - Nonreduced
Lane 5	SeeBlue Plus 2 MW Marker
Lane 6	0.0% Tween 80 - Reduced
Lane 7	0.01% Tween 80 - Reduced
Lane 8	0.05% Tween 80 - Reduced
Lane 9	0.1% Tween 80 - Reduced
Type of Gel - 4-12% Bis-Tris	

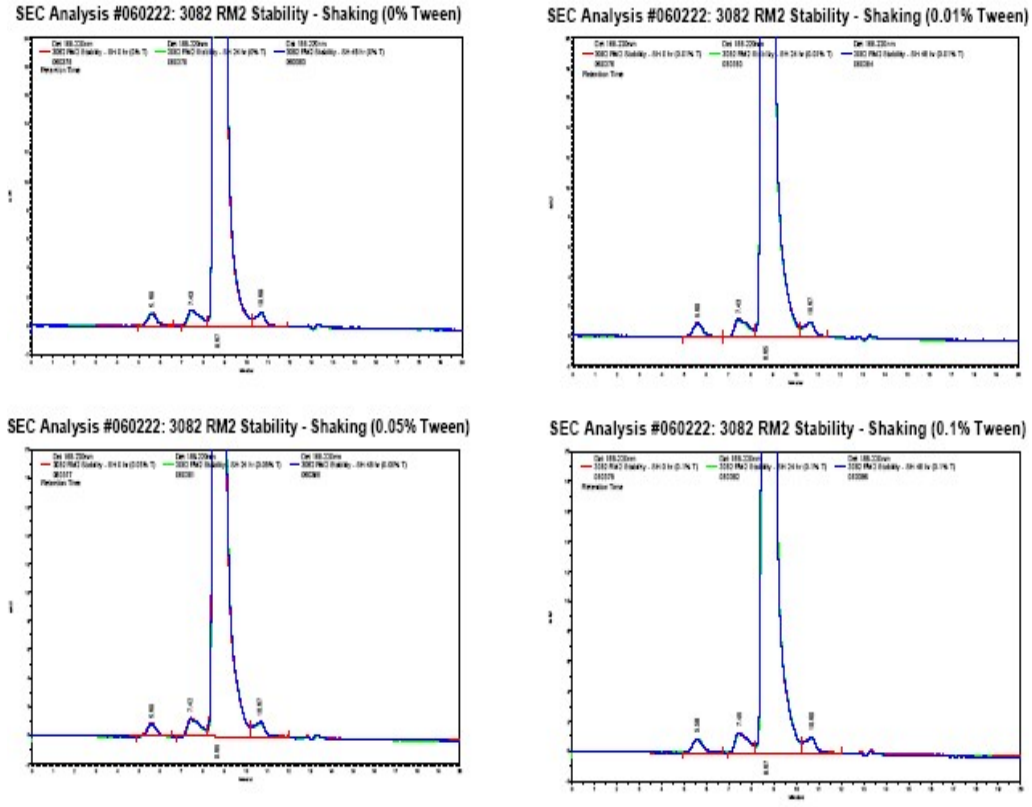
The SDS-PAGE analysis shows no significant differences occurring to the product during the storage at 40°C and there is no apparent difference between the Tween 80 concentrations used in this study.

Fig. 9: SDS – PAGE analysis- images of RM1humanized mAb‟s at Accelerated temperature condition (40°C) stability study on different Tween -80 concentration formulations.

3.6 Stability Study of RM2 humanized mAb's

3.6.1 Shaking Stability Study of RM2, SEC-HPLC Analysis of Aggregates

The SEC-HPLC chromatograms for this study are shown below:



Summary of SEC HPLC Traces

RM2	% Aggregate			
% Tween 80 (w/v)	0.00	0.01	0.05	0.10
0 hr	1.0	1.0	1.0	1.0
24 hr	0.9	1.0	1.0	1.0
48 hr	1.0	1.0	1.0	1.1

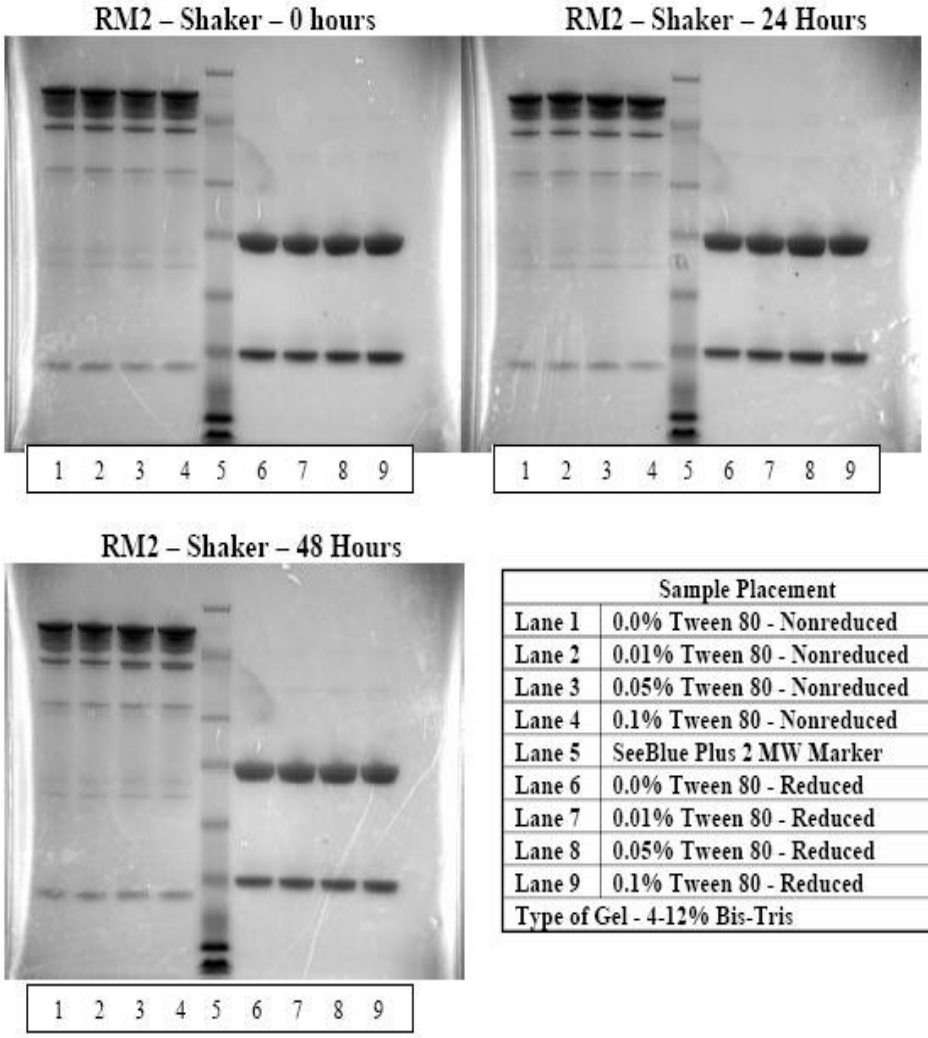
Analysis-SEC 060222

The level of aggregate was not seen to increase during the study and / or across the range of Tween 80 concentrations used in this study.

Fig. 10: Chromatograms of SEC analysis of RM2 humanized mAb‟s on different Shaking times as part of stability study for 48 hours on different Tween -80 concentration formulations.

3.6.2 Shaking stability of different Tween- 80formulations of RM2mAb's using SDS-PAGE (Reducing and Non Reducing)

The SDS-PAGE analysis of the samples is shown below for the three incubation times.



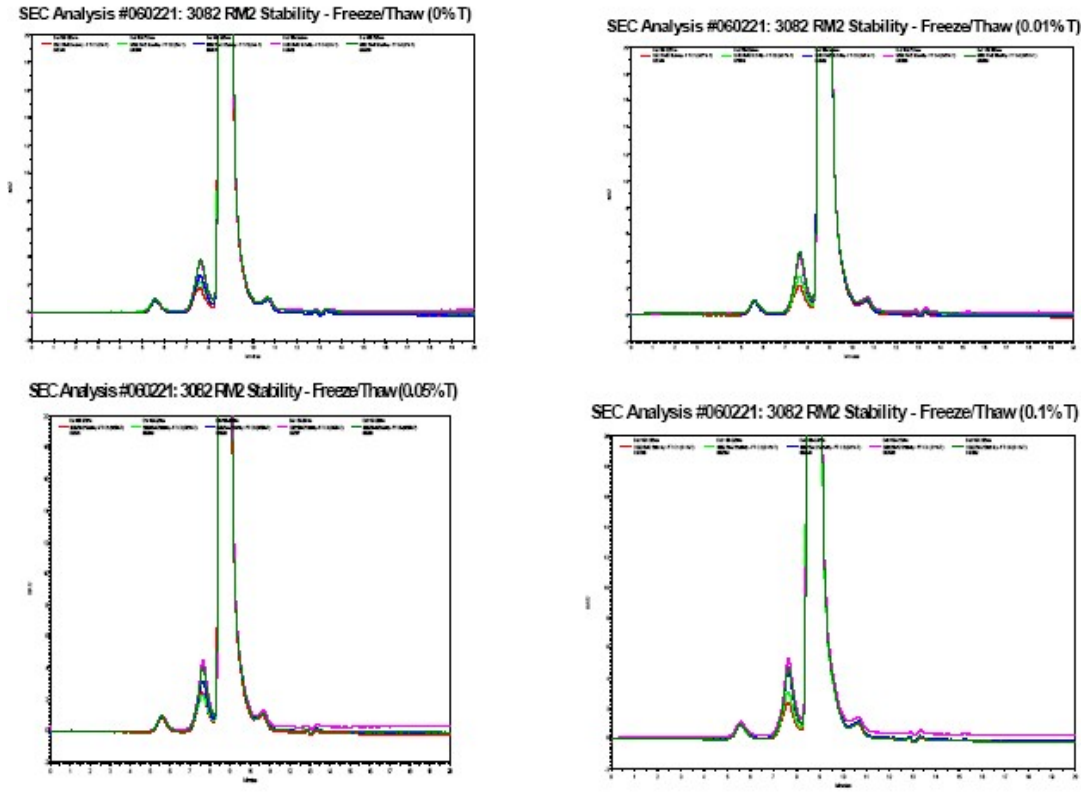
Sample Placement	
Lane 1	0.0% Tween 80 - Nonreduced
Lane 2	0.01% Tween 80 - Nonreduced
Lane 3	0.05% Tween 80 - Nonreduced
Lane 4	0.1% Tween 80 - Nonreduced
Lane 5	SeeBlue Plus 2 MW Marker
Lane 6	0.0% Tween 80 - Reduced
Lane 7	0.01% Tween 80 - Reduced
Lane 8	0.05% Tween 80 - Reduced
Lane 9	0.1% Tween 80 - Reduced
Type of Gel - 4-12% Bis-Tris	

The SDS-PAGE analysis shows no significant difference occurring to the product during the study and / or across the range of Tween 80 concentrations used in this study.

Fig. 11: SDS – PAGE analysis images of RM2 humanized mAbs on different Shaking times as part of stability studies for 48 hours on different Tween -80 concentration formulations.

3.6.3 Freeze/Thaw Stability Study of RM2, SEC-HPLC Analysis of Aggregates

The SEC-HPLC chromatograms for this study are shown below:



Summary of SEC HPLC Traces

RM2	% (w/v) Tween 80			
Cycle	0.00	0.01	0.05	0.10
1	1.4	1.5	1.6	1.5
2	1.6	1.8	1.5	1.8
3	1.8	2.3	1.8	2.2
4	2.3	2.4	2.4	2.6
5	2.3	2.5	2.2	2.5

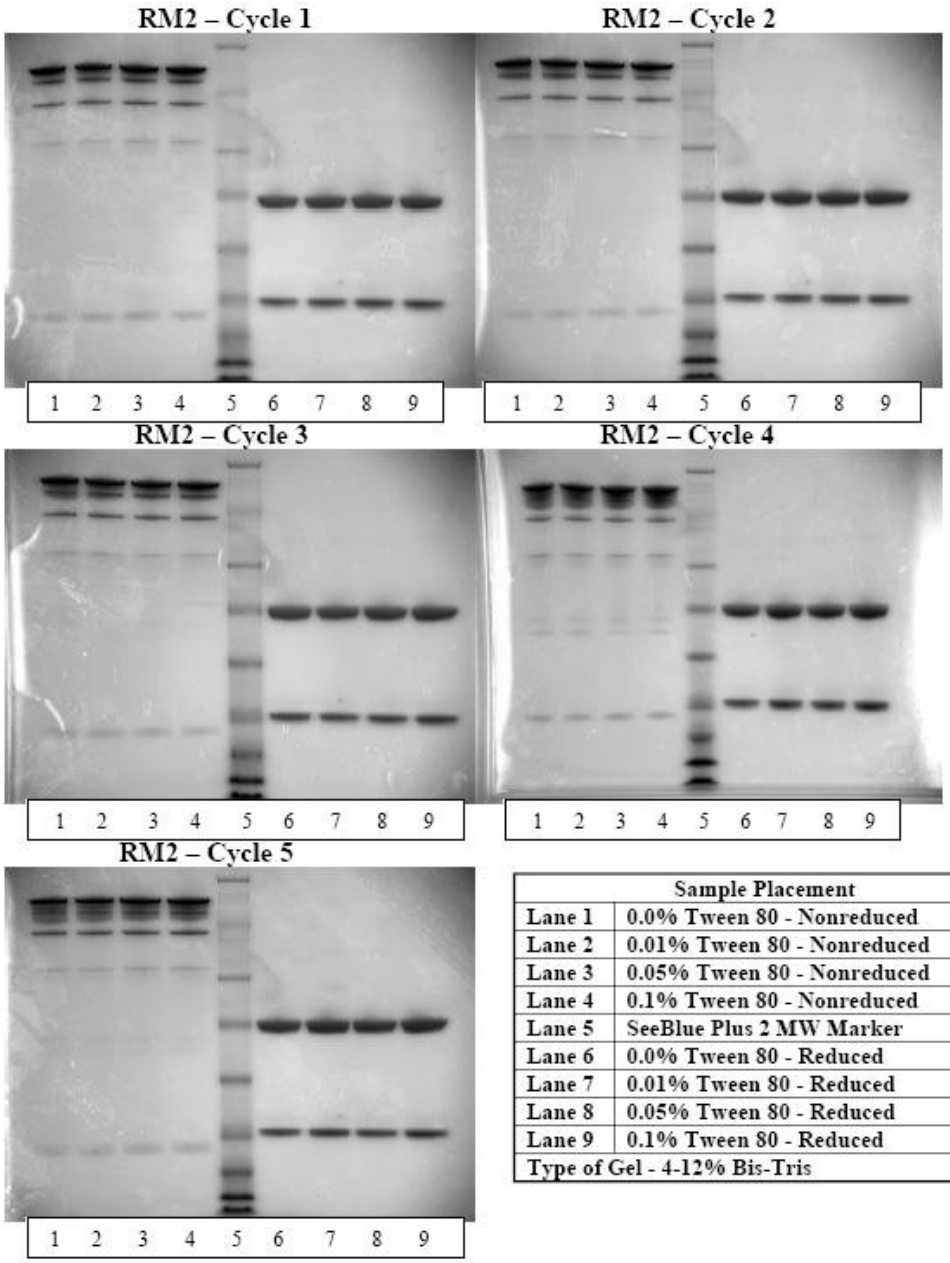
Analysis-SEC 060221

The level of aggregate appeared to increase as the number of freeze / thaw cycles was increased, although the level was below 3%. This increase in aggregate appeared to be independent of the Tween 80 concentrations in the formulation.

Fig. 12: Chromatograms of SEC analysis of RM2 humanized mAb‟s on different Freeze/Thawing (5 cycle times) as part of stability study on different Tween -80 concentration formulations.

3.6.4 Freeze/Thaw Stability Study of different Tween- 80formulations of RM2 mAb' s by SDS-PAGE Analysis

The SDS-PAGE analysis of the samples is shown below for the five freeze /thaw cycles.



Sample Placement	
Lane 1	0.0% Tween 80 - Nonreduced
Lane 2	0.01% Tween 80 - Nonreduced
Lane 3	0.05% Tween 80 - Nonreduced
Lane 4	0.1% Tween 80 - Nonreduced
Lane 5	SeeBlue Plus 2 MW Marker
Lane 6	0.0% Tween 80 - Reduced
Lane 7	0.01% Tween 80 - Reduced
Lane 8	0.05% Tween 80 - Reduced
Lane 9	0.1% Tween 80 - Reduced
Type of Gel - 4-12% Bis-Tris	

The SDS-PAGE analysis shows no significant differences occurring to the product during the multiple freeze / thaw cycles and / or across the range of Tween 80 concentrations used in this study.

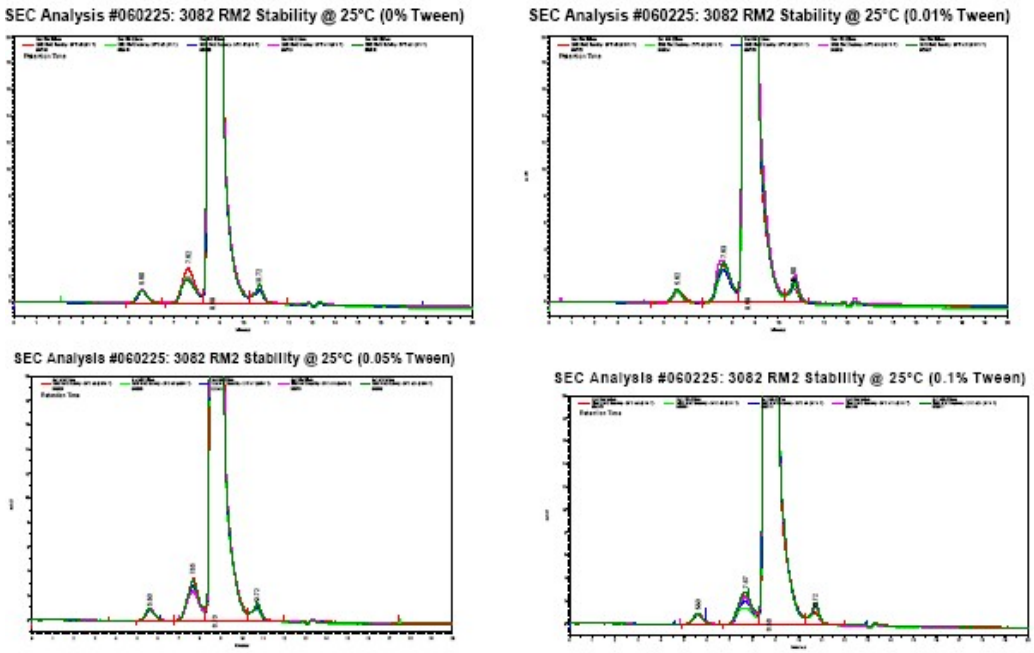
Fig. 13: SDS – PAGE analysis images of RM1humanized mAb‟s of different Freeze thawing cycles (5 cycles) as part of stability study on different Tween -80 concentration formulations.

3.7Accelerated Stability Study results of RM2 h~mAb

3.7.1Room Temperature Stability of different Tween- 80formulations of RM2 h~mAb at accelerated condition 25°C using SEC-HPLC analysis

The SEC-HPLC chromatograms for this study are shown below:

25°C



Summary of SEC HPLC Traces

RM2	% aggregate			
Temp (°C)	25	25	25	25
% Tween 80 (w/v)	0	0.01	0.05	0.1
0	1.7	1.7	1.9	1.6
3	1.4	1.6	1.6	1.2
7	1.4	1.7	1.7	1.5
14	1.4	1.8	1.5	1.6
21	1.4	1.9	1.9	1.8

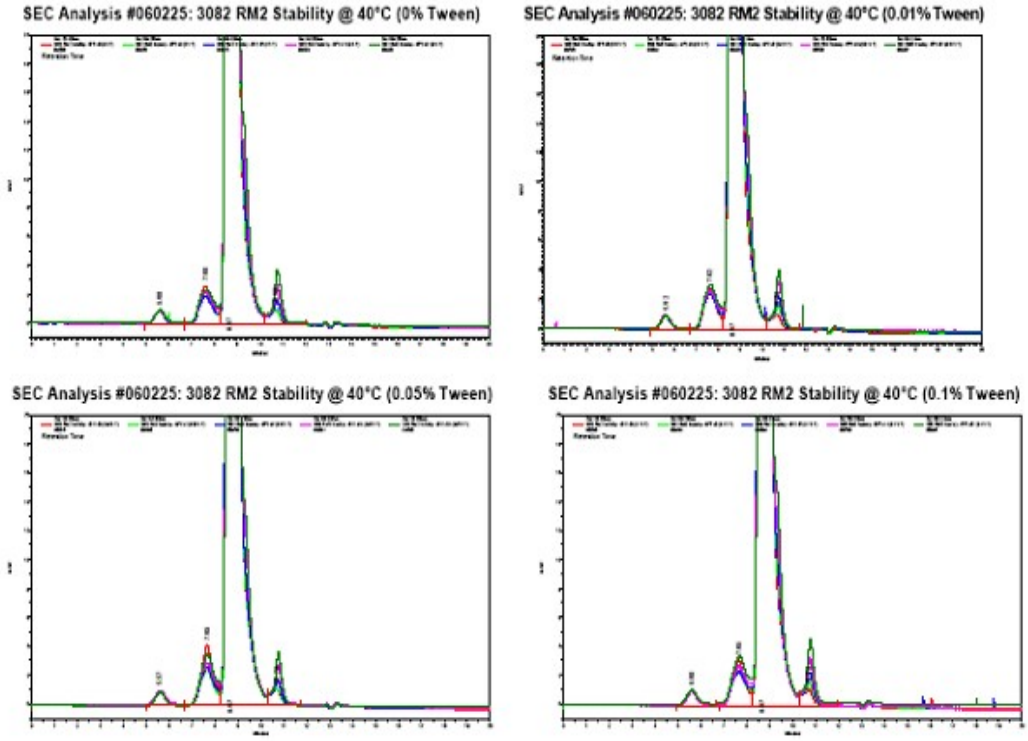
The level of aggregate was not seen to significantly increase during the study and / or across the range of Tween 80 concentrations used in this study.

Figure-14: Chromatograms of SEC analysis of RM2 humanized mAb‟s at Room temperature (25°C) stability study on different Tween -80 concentration formulations.

3.7.2Accelerated Stability of different Tween- 80 formulations of RM2mAb's using SEC-HPLC Analysis of Aggregates.

The SEC-HPLC chromatograms for this study are shown below:

40°C



Summary of SEC HPLC Traces

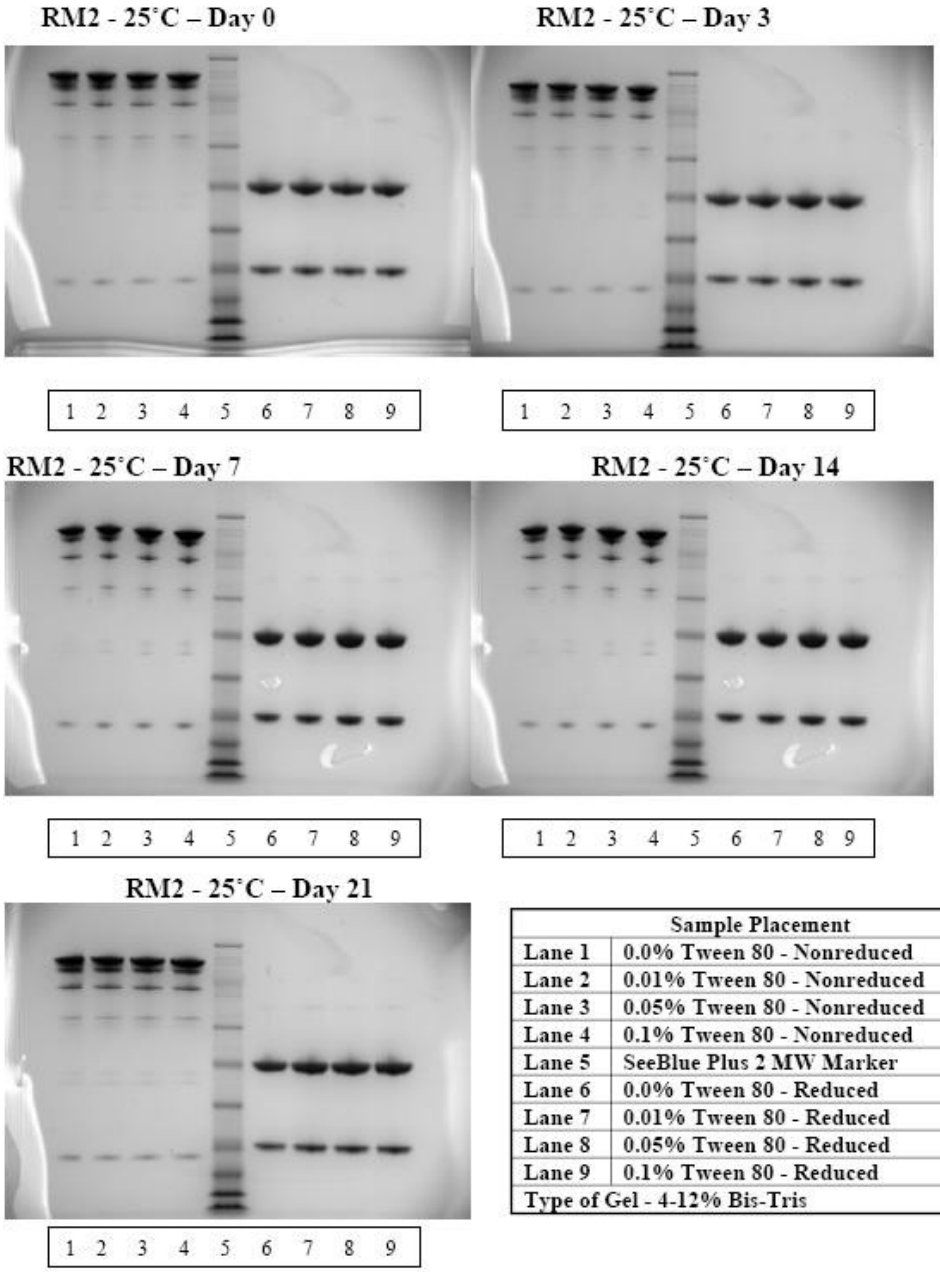
RM2	% aggregate			
Temp (°C)	40	40	40	40
% Tween 80 (w/v)	0	0.01	0.05	0.1
0	1.7	1.7	2.1	1.8
3	1.5	1.7	1.6	1.7
7	1.5	1.7	1.7	1.7
14	1.7	1.8	1.8	1.8
21	1.7	2.0	2.2	2.1

The level of aggregate was not seen to significantly increase during the study and / or across the range of Tween 80 concentrations used in this study. However the level of an apparent degradant does appear to increase and this was observed for all Tween 80 concentrations.

Figure-15: Chromatograms of SEC analysis of RM2 humanized mAb‟s at Accelerated temperature (40°C) stability condition study on different Tween -80 concentration formulations.

3.7.3 Room Temperature (25°C) Stability of different Tween- 80 formulations of RM2 h~mAb's using SDS-PAGE Analysis for 21 days stability (5 time points.

The SDS-PAGE analysis of the samples is shown below for the five incubation times.

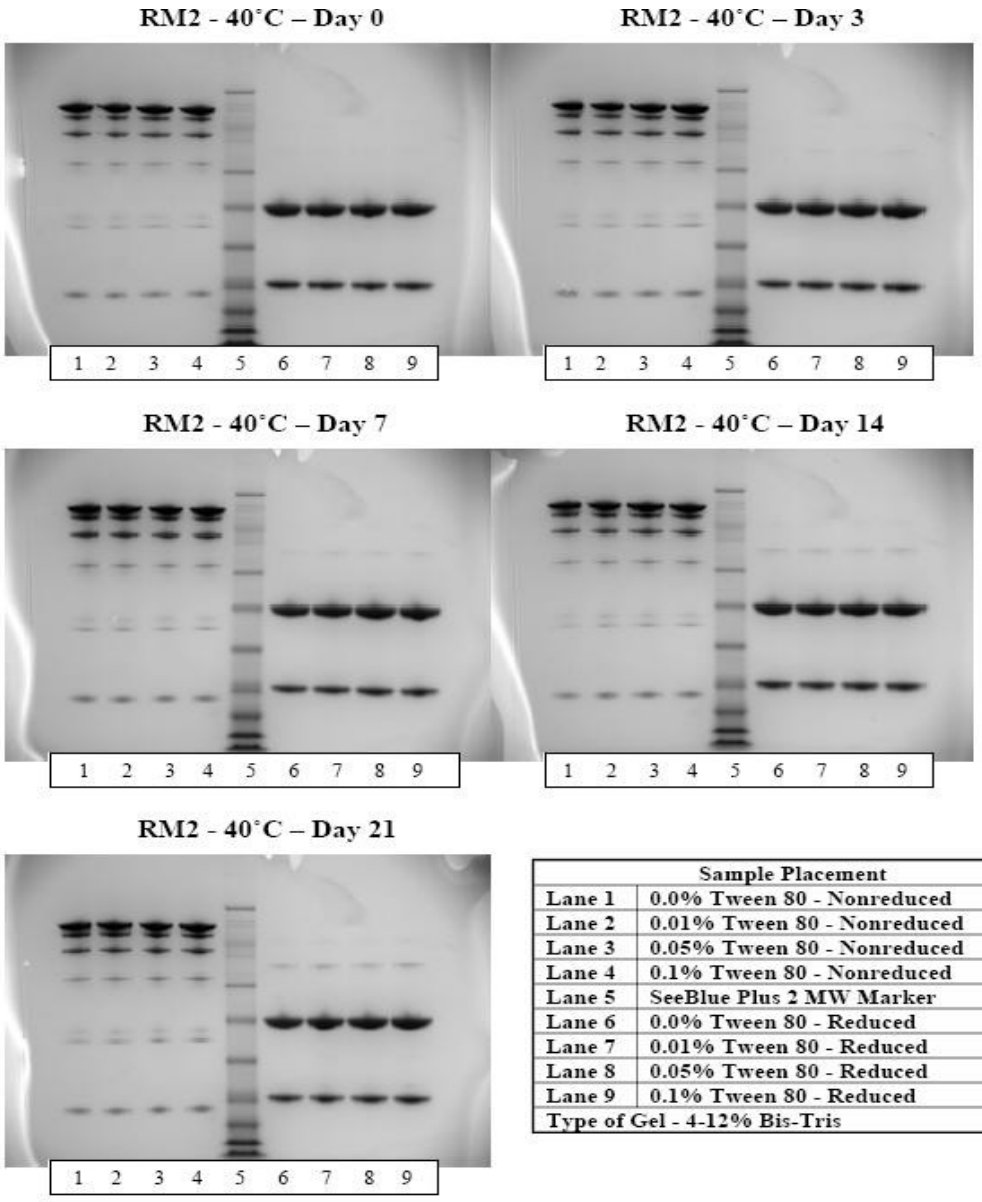


Sample Placement	
Lane 1	0.0% Tween 80 - Nonreduced
Lane 2	0.01% Tween 80 - Nonreduced
Lane 3	0.05% Tween 80 - Nonreduced
Lane 4	0.1% Tween 80 - Nonreduced
Lane 5	SeeBlue Plus 2 MW Marker
Lane 6	0.0% Tween 80 - Reduced
Lane 7	0.01% Tween 80 - Reduced
Lane 8	0.05% Tween 80 - Reduced
Lane 9	0.1% Tween 80 - Reduced
Type of Gel - 4-12% Bis-Tris	

The SDS-PAGE analysis shows no significant differences occurring to the product during the study and / or across the range of Tween 80 concentrations used in this study.

Figure-16: SDS – PAGE analysis- images of RM1humanized mAb‟s at Room temperature (25°C) stability study on different Tween -80 concentration formulations.

3.7.4Accelerated Temperature (40°C) Stability of different Tween- 80 formulations of RM2 h~mAb's using SDS-PAGE Analysis for 21 days stability (5 time points)



Sample Placement	
Lane 1	0.0% Tween 80 - Nonreduced
Lane 2	0.01% Tween 80 - Nonreduced
Lane 3	0.05% Tween 80 - Nonreduced
Lane 4	0.1% Tween 80 - Nonreduced
Lane 5	SeeBlue Plus 2 MW Marker
Lane 6	0.0% Tween 80 - Reduced
Lane 7	0.01% Tween 80 - Reduced
Lane 8	0.05% Tween 80 - Reduced
Lane 9	0.1% Tween 80 - Reduced
Type of Gel - 4-12% Bis-Tris	

The SDS-PAGE analysis shows no significant differences occurring to the product during the study and / or across the range of Tween 80 concentrations used in this study.

Figure-17: SDS – PAGE analysis- images of RM1humanized mAb‟s at Accelerated temperature condition (40°C) stability study on different Tween -80 concentration formulations

5.CONCLUSION

RM1 was stable under the shaking conditions and in extended storage at 25°C. Multiple freeze / thaw cycles increased the level of antibody aggregation and this seemed to be increased by the presence of Tween 80 in the formulation buffer. Extended incubation at 40°C resulted in the increase of a degradation product (as determined by HPLC). This was not detected by the other analysis methodologies.

RM2 was stable under the shaking conditions and in extended storage at 25°C. Multiple freeze / thaw cycles showed an apparent increase in the level of antibody aggregation, although the levels were below 3%. This increase seemed independent of the level of Tween 80 in the formulation buffer. Extended incubation at 40°C resulted in the increase of a degradation product (as determined by HPLC). This was not detected by the other analysis methodologies.

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