インスリン イコデク（遺伝子組換え）

2.7.1 生物薬剤学試験及び関連する分析法

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略語一覧

AUC	:	Area under curve（曲線下面積）
CFR	:	Code of Federal Regulations
CI	:	Confidence interval（信頼区間）
CP	:	cut point
CR	:	cross reactivity（交叉反応性）
%CV	:	% coefficient of variation（%変動係数）
EIRR	:	Estimated injection region ratios
ELISA	:	enzyme-linked immunosorbent assay（酵素免疫吸着測定法）
ISR	:	incurred sample re-analysis（投与後試料の再分析による定量値の再現性確認）
LOCI	:	Luminescence Oxygen Channeling Immunoassay（発光酸素チャネリング免疫測定法）
MRD	:	minimum required dilution（最小希釈倍率）
NF	:	normalisation factor（正規化係数）
NSCP	:	normalised screening cut point（正規化スクリーニングカットポイント）
NTCP	:	normalised titre cut point（正規化抗体価カットポイント）
OD	:	optical density（光学密度）
QC	:	quality control
RIA	:	radioimmunoassay（ラジオイムノアッセイ）
RT	:	room temperature（室温）
SD	:	single dose（単回投与）
$t_{1/2}$	:	terminal half-life（終末相半減期）
T2D	:	type 2 diabetes（2 型糖尿病）
TMB	:	3,3’,5,5’-Tetramethylbenzidine

臨床試験の表記：
本概括評価では、個々の臨床試験（NN1436-xxxx）を「xxxx 試験」と表記する（例：「NN1436-4572」の場合「4572 試験」と表記する）。

2.7.1.1　背景及び概観

インスリン イコデクは、ヒトインスリンの B 鎖 30 位のスレオニンを欠失させ、A 鎖 14 位のチロシンをグルタミン酸に置換し、B 鎖 16 位のチロシン及び B 鎖 25 位のフェニルアラニンをヒスチジンに置換した、作用が長時間持続するヒトインスリンアナログである。側鎖は、B 鎖 29 位のリジンの側鎖のアミノ基を介してペプチド骨格と結合している。

本プロジェクトの目的は、糖尿病患者への週 1 回皮下投与に適した安全かつ有効な Basal インスリン製剤を開発することであった。インスリン イコデクは作用が長時間持続する新規の Basal インスリンアナログであり、終末相の消失半減期（$t_{1/2}$）は約 196 時間である。本薬はペプチド骨格と脂肪酸を含む側鎖からなる分子である。ペプチド骨格は、修飾によりタンパク質分解を受けにくく、かつヒトインスリンよりもインスリン受容体への親和性が低下している。また、アシル化された長い側鎖によりアルブミンと強く結合する。これら 3 つの特性すべてが、インスリン イコデクの作用の持続化に寄与している。インスリン イコデクは 700 単位/mL に相当する 4200 nmol/mL 溶液として製剤化されており、インスリン イコデク分子は 1 日 1 回投与の Basal インスリンと同等の力価を有すると考えられる。

本概要では、インスリン イコデク測定に用いた生体試料中薬物濃度分析法の概観を記述する。

2.7.1.1.1　製剤開発

インスリン イコデク 700 単位/mL 製剤の組成及び各成分の配合目的を表 2.7.1.1-1 に示す。本製剤は、2022 年 9 月 19 日の cut-off 時点で完了した臨床試験及び進行中の臨床試験において使用した製剤であり、市販予定の製剤でもある。

インスリン イコデク 700 単位/mL 製剤は 4200 nmol/mL のインスリン イコデクを含む。添加剤はすべて非動物由来かつ医薬品グレードであり、市販のインスリン注射剤に広く使用されている。本製剤はフェノール及び m-クレゾールを防腐剤として、グリセロール及び塩化ナトリウムを等張化剤として含有する。本製剤の保存効力試験の結果、インスリン イコデク 700 単位/mL 製剤は米国薬局方の判定基準及び欧州薬局方 criteria B に適合している。亜鉛は安定性を確保するため添加されている。さらに、亜鉛はインスリンのオリゴマー形成作用を有しており、この作用が薬物動態プロファイルに影響を及ぼす。本製剤の pH は 7.4 である。

表 2.7.1.1-1　　インスリン イコデクの製剤組成

Component	Quantity per ml	Function
Active ingredient		
Insulin icodec drug substance	4200 nmol	Active pharmaceutical ingredient
Excipients		
Zinc (as zinc acetate)	101 µg	Stabilising agent
Phenol	5.65 mg[a]	Stabilising agent, Preservative
Metacresol	1.08 mg[a]	Preservative
Glycerol	15 mg	Tonicity agent
Sodium chloride	1.17 mg	Tonicity agent
Hydrochloric acid	q.s.[b]	pH adjustment
Sodium hydroxide	q.s.[b]	pH adjustment
Water for injections	To make 1 ml	Solvent

[a] An overage of [redacted] is added to compensate for loss during manufacturing
[b] To reach pH 7.4

2.7.1.1.2　バイオアベイラビリティ及び生物学的同等性を検討する試験の根拠及び全般的なアプローチ

今回の承認申請において申請資料に含めたすべての臨床試験は市販予定の 700 単位/mL 製剤を用いて実施しているため、生物学的同等性試験は不要であった。

1 つの臨床試験（4572 試験）において、腹部、上腕部及び大腿部へインスリン イコデクを皮下投与した時の相対的バイオアベイラビリティを検討した。主な結果の要約を 2.7.1.2 項に示す。

4572 試験は、以下のガイドラインの推奨に従い実施した。

- 米国食品医薬品局：Code of Federal Regulations (CFR) Title 21 CFR Part 320 Bioavailability and Bioequivalence Requirements, April 2012.[1]
- 欧州医薬品庁：Note for Guidance on the Investigation of Bioavailability and Bioequivalence (CPMP/EWP/QWP/1401/98), Rev. 1, January 2010.[2]

4572 試験の治験総括報告書は Module 5.3.1.1 に示す。

2.7.1.1.3　測定方法

2.7.1.1.3.1　　インスリン イコデクの測定

ヒト血清中及びヒト尿中インスリン イコデク濃度は、発光酸素チャネリング免疫測定法（LOCI 法、AlphaLISA 法とも呼ばれる）により定量した。このインスリン イコデク測定法は現行のガイドラインに従ってバリデートされ、同ガイドラインの判定基準を満たした。

バイオアナリシスのライフサイクル情報（Bioanalytical Method Life Cycle Information）の概観を、バリデーションについては付録 2.7.1.4.1、Table 4.1.1 に、臨床試験については付録 2.7.1.4.1、Table 4.1.2 に示す。測定法バリデーション及び臨床試験の「測定方法の性能の要約（Summary Method

Performance）」を付録 2.7.1.4.1、Table 4.1.3 に、「測定方法の変更及びクロスバリデーションの結果の要約（Summary of Method Modifications and Cross-Validation Results）」を付録 2.7.1.4.1、Table 4.1.4 に示す。

個々のバリデーション報告書を Module 5.3.1.4 に示す。各臨床試験の個別の生体試料中薬物濃度分析報告書（Bioanalytical report）は、各試験の治験総括報告書の Appendix 16.1.10 に示す。

2.7.1.1.3.1.1　　測定法の詳細

臨床試験試料中のインスリン イコデクを測定するための薬物動態測定法は、ノボ ノルディスク社が開発及びバリデーションを実施した。この測定法は、2 種類のラテックスビーズ試薬（アクセプタービーズ及びドナービーズ）の近接を利用した LOCI 法である。ドナービーズは非特異的で、ストレプトアビジンでコーティングされており、インスリン イコデクの B 鎖 25 位の置換したヒスチジンに対して特異的なビオチン化抗体（M-GIIone-2F4A80）に結合する。アクセプタービーズにはモノクローナル抗体（HUI-018）が結合しており、ヒトインスリンを認識する。この 3 種類の反応物がインスリン イコデク存在下で近接状態となり、ビーズ－凝集体－免疫複合体を形成する。ドナービーズ中に存在する光感受性物質が励起されることにより周囲の酸素が一重項酸素に変換され、アクセプタービーズと反応して化学発光が引き起こされる。カウント単位（cps）で示される発光量は、インスリン イコデク濃度に比例する。

測定用試料中のインスリン イコデク濃度は、分析単位ごとに含めた一連の検量線用試料の測定結果から内挿して定量した。検量線用試料及び QC 試料は測定対象試料（ヒト血清又は尿）と同一のマトリックスを用いて調製した。1 試料あたり 2 回測定し、2 回の平均濃度を報告した。

2.7.1.1.3.1.2　　バリデーションの評価項目

測定法のバリデーションは現行のガイドライン [3, 4] に従って実施された。

臨床試験試料のヒト血清中インスリン イコデク測定に使用した LOCI 法は、当初はノボ ノルディスク社が開発し、フルバリデーションを実施し（Module 5.3.1.4、study 212348 及び study 212408）、最小希釈倍率（MRD）を 10 倍、測定範囲を 200～50000 pmol/L に設定した（A 法）。本測定法をさらに最適化し（Module 5.3.1.4、study 216306 及び study 300072）、MRD を 50 倍、新しい測定範囲を 500～100000 pmol/L（上限は 150000 pmol/L から切り下げ）に設定した（B 法）。本測定法のフルバリデーションは、Celerion 社（スイス、チューリッヒ）（Module 5.3.1.4、study 306660-CA25310-01 及び study 306660-CA25310-02）及び Wuxi Apptec 社（中国、上海）（Module 5.3.1.4、study 321497）においても実施し、測定範囲を 500～80000 pmol/L に設定した（C 法）。

臨床試験試料のヒト尿中インスリン イコデク測定に使用される LOCI 法の開発及びフルバリデーションは Celerion 社（スイス）（Module 5.3.1.4、study 306661-CA25312-01 及び study 306661-CA25312-02、D 法）において実施した。

バリデーションを実施した測定の評価項目は、真度、精度、希釈直線性、フック効果（プロゾーン）、選択性、特異性、平行性、自動化及び安定性であった。バリデーション試験結果の要約を付録 2.7.1.4.1、Table 4.1.3 に示す。

インスリン イコデクの臨床開発プログラムにおける試料測定は、ノボ ノルディスク社、Celerion 社（スイス、チューリッヒ）及び Wuxi Apptec 社（上海、中国）で実施し、インスリン イコデク測定の再現性は、健康被験者及び特別な患者集団（1 型糖尿病及び 2 型糖尿病患者、肝障害を有する被験者及び腎障害を有する被験者ならびに日本人被験者及び中国人被験者）から得られた試料における評価を含め、すべての臨床試験において incurred sample re-analysis（ISR）を実施することにより評価した。ISR に用いる試料数は、生体試料中薬物濃度分析法のバリデーションに関する欧州医薬品庁及び米国食品医薬品局のガイダンス [3, 4] に基づき、最初の 1000 試料の 10%に、1000 を超えた試料数の 5%を加えた数とした。すべての ISR 評価が判定基準の範囲内（すなわち、ISR 対象試料の 3 分の 2 以上が初回測定値の±30%以内）であったため、測定法は再現性があると判断した。ISR の結果は個々の生体試料中薬物濃度分析報告書に示す。測定結果は個々の治験総括報告書の Appendix 16.1.10 に示す（付録 2.7.1.4.1 の一覧を参照）。

2.7.1.1.3.2　抗体測定

抗体測定の詳細については、Integrated summary of immunogenicity（Module 5.3.5.3）に示す。

生体試料分析における段階的アプローチ

インスリン イコデクに結合する抗体及びヒトインスリンに対する交叉反応性は、バリデートされたラジオイムノアッセイ（RIA）法を用いて評価した。第 1 相試験の 4314 試験を除くすべての臨床試験において、各試料の抗インスリン イコデク抗体の陽性／陰性を判定し、陽性の場合は抗体価を求め、ヒトインスリンに対する交叉反応性の陽性／陰性を判定した。

4314 試験では、カットポイントを設定しない subtraction RIA 法を用いた（バリデーション報告書：Module 5.3.1.4、study 212290）。さらに 4314 試験のみ、抗インスリン イコデク抗体の IgE サブタイプをバリデートされたイムノキャップ法で測定し、IgG4 サブタイプをバリデートされた酵素免疫吸着測定法（ELISA）で測定した。

インスリン イコデクによる抗体誘導が及ぼす臨床的に問題となる影響を検出するため、抗体価と、関連する有効性及び安全性のパラメータ（HbA1c、インスリン投与量、低血糖及び過敏症反応など）との相関を 1 つの第 2 相試験（4383 試験）及び 4 つの第 3 相試験（4478 試験、4479 試験、4480 試験及び 4625 試験）において検討した。抗インスリン抗体価と、関連する安全性及び有効性パラメータとの相関は、製剤の有効性及び患者の安全性を評価するための臨床試験評価項目の一部であった〔Integrated summary of immunogenicity（Module 5.3.5.3）〕。

測定法

<u>カットポイントを設定した抗インスリン イコデク結合抗体測定法</u>

4314 試験を除くすべての臨床試験の抗インスリン イコデク結合抗体の RIA 法のバリデーションを、主要バリデーション（主要バリデーション報告書：Module 5.3.1.4、study 301510）については 2019 年に、追加バリデーション（追加バリデーション報告書：Module 5.3.1.4、study 321724）については 2021 年に Celerion 社（スイス）において実施した。本測定法のバリデーションの要約及び主なバリデーション結果（表 2.7.1.1-2 及び表 2.7.1.1-3）を以下に示す。

バリデーション試料をグリシン塩酸塩とインキュベートして、試料中に存在する抗体と干渉物質となる可能性のある薬物との結合を解離させた。存在するすべての抗薬物抗体を中和用 Tris 緩衝液中でポリエチレングリコールにより沈殿させた。沈殿物を再溶解して、5°C で一晩インキュベートすることにより、試料中のインスリン イコデクに対する抗体をインスリン イコデクのトレーサーに結合させた。翌日、ポリエチレングリコールを用いて、抗体に結合している可能性のある抗原とともに免疫グロブリンを沈殿させた。上清を除去後、沈殿物をガンマカウンターで測定した。放射能は添加した総放射能に対する比率（%B/T）として示した。試料の%B/T 値は、試料中の抗インスリン イコデク抗体の半定量的測定値である。

スクリーニング分析では、非標識薬物の非存在下でバリデーション試料を測定した（系列 A）。バリデーションカットポイント（vCP）は、カットポイント設定用測定を行って決定し、正規化係数（NF）算出のために用いた。偽陽性率を 5%とした場合の NF の値 1.18 を正規化スクリーニングカットポイント（NSCP）の算出に用いた。この NSCP は分析単位ごとに固有の値である。スクリーニング分析において、試料を NSCP「以上」又は NSCP「未満」に分類した。

確認分析において、試料を非標識薬物の非存在下（系列 A）及び過剰量の非標識（cold）インスリン イコデク存在下（系列 B）で測定した。第 3 相試験前に本測定法を最適化した後、追加バリデーション試験において特異性／阻害カットポイント（Inhibition CP、iCP）を再度決定したところ 20.03%であった（表 2.7.1.1-3）。%B/T 値が NSCP 以上かつ系列 A 及び系列 B における阻害率の差（%InhibitionA-B）が iCP 以上の試料を抗インスリン イコデク抗体陽性とした。確認分析において%B/T 値が NSCP 未満又は%InhibitionA-B の値が iCP 未満の試料を陰性とした。

交叉反応分析において、試料を非標識薬物の非存在下（系列 A）及びヒトインスリン存在下（系列 C）で測定した。交叉反応性カットポイント（CR CP）はこのバリデーション試験において決定され、23.09%であった（表 2.7.1.1-2）。%B/T 値が NSCP 以上かつ系列 A 及び系列 C における阻害率の差（%InhibitionA-C）が CR CP 以上の試料をヒトインスリンに対する交叉反応陽性とした。%InhibitionA-C の値が CR CP 未満の試料をヒトインスリンに対する交叉反応陰性とした。

抗体価分析において、試料の希釈後に ^{125}I 標識インスリン イコデクのみを添加して、スクリーニング分析（系列 A）と同様に測定した。抗体価分析では、試料を正規化抗体価カットポイント（NTCP）「以上」又は NTCP「未満」に分類した。個別の測定試験計画における規定に従い、偽陽性率を 1%又は 0.1%とした場合の NF を用いて、分析バッチごとに NTCP を決定した。追加バリデーション前（第 3 相試験前）は、インスリン治療歴のない 2 型糖尿病患者の血清で力価（希釈倍率）を検討したが、その後の試験では健康なヒト血清で力価（希釈倍率）を検討した（表 2.7.1.1-2）。

表 2.7.1.1-2　抗インスリン イコデク結合抗体のラジオイムノアッセイのカットポイント設定を伴う主要バリデーションで得られた主な結果（Module 5.3.1.4、study 301510）

Parameters	**Evaluation**	**Result**
Screening cut point (vCP) (1.0% false positive)	median($Binding_{MeanA}$) + 2.326 x MADn($Binding_{MeanA}$)	1.79 [%B/T]
(5.0% false positive)	median($Binding_{MeanA}$) + 1.645 x MADn($Binding_{MeanA}$)	1.66 [%B/T]
Normalisation factor (NF)		

Parameters	Evaluation	Result
	vCP with 1.0%/Mean of QC_0 $Binding_{MeanA}$ vCP with 5.0%/Mean of QC_0 $Binding_{MeanA}$	1.27 1.18
Normalised screening cut point (5.0 % false positive)	1.18 [%B/T] *Mean of QC_0 $Binding_{MeanA}$	Analytical run specific
Normalised titre cut point (1.0 % false positive)	1.27 [%B/T] *Mean of QC_0 $Binding_{MeanA}$	Analytical run specific
Confirmatory cut point (1.0 % false positive)	median($Binding_{MeanA}$) + 2.326 x MADn($Binding_{MeanA}$)	21.23 %
Cross-reactivity cut point (1.0 % false positive)	median($Binding_{MeanA}$) + 2.326 x MADn($Binding_{MeanA}$)	23.09 %
Assay sensitivity for anti-insulin 0287 antibodies	Screening sensitivity at: 99.0 % confidence level 95.0 % confidence level	 3.94 ng/mL 2.81 ng/mL
	Confirmatory sensitivity at: 99.0 % confidence level 95.0 % confidence level	 6.27 ng/mL 4.86 ng/mL
Assay sensitivity anti-human insulin (HUI-018) antibodies	Screening sensitivity at: 99.0 % confidence level 95.0 % confidence level	 39.30 ng/mL 28.99 ng/mL
Drug interference	For: 0.20 nmol/mL of Insulin 0287 1.20 nmol/mL of Insulin 0287	 4.00 ng/mL of anti-Insulin 0287 antibodies 160.00 ng/mL of Insulin 0287 antibodies
Drug tolerance[4]	For: 100.00 ng/mL of anti-Insulin 0287 antibodies 50.00 ng/mL of anti-Insulin 0287 antibodies 10.00 ng/mL of anti-Insulin 0287 antibodies 3.94 ng/mL of anti-Insulin 0287 antibodies	 1.00 nmol/mL of Insulin 0287 1.00 nmol/mL of Insulin 0287 0.02 nmol/mL of Insulin 0287 0.00 nmol/mL of Insulin 0287
Recovery (individual serum)	For low spike (3.94 ng/mL) ≥ NSCP For low spike (5.91 ng/mL) + 50% ≥ NSCP For high spike (500.00 ng/mL) within ± 20% of pool	10/10 10/10 10/10
Haemolysis effect[5]	For low spike (2.5 ng/mL) For high spike (500.00 ng/mL)	no impact up to 5% no impact up to 5%
Lipaemic effect[5]	For low spike (2.5 ng/mL) For high spike (500.00 ng/mL)	no impact no impact
FT and benchtop stability after storage at -20°C[6]	For negative (0.00 ng/mL) For low spikes (3.94 and 6.27 ng/mL) For medium spike (50.00 ng/mL) For high spike (500.00 ng/mL)	For all samples tested: 10 cycles, 34.3 hrs at RT
FT and benchtop stability after storage at -20°C[6] (anti-human insulin antibodies HUI-018)	For negative (0.00 µg/mL) For low spike (39.30 ng/mL) For high spike (5.00 µg/mL)	For all samples tested: 10 cycles, 34.3 hrs at RT
Titre precision	%CV at NTCP with 1% failure rate	21.3
MSR	$10^{t(0.05,df)*\sqrt{2}*SD}$ $10^{t(0.95,df)*\sqrt{2}*SD}$	0.56 1.80

Parameters	**Evaluation/Results**						
Assay evaluation with QCs levels	QC_0	QC_{Low_s}	$QC_{Low_s_CR}$	QC_{Low_i}	QC_{Med}	QC_{High}	QC_{High_CR}
Reference item used	A	A	B	A	A	A	B
Matrix used	T2D	T2D	T2D	T2D	Healthy	Healthy	Healthy
Spiking concentration	0.00 ng/mL	3.94 ng/mL	39.30 ng/mL	6.27 ng/mL	50.00 ng/mL	500.00 ng/mL	5.00 µg/mL
QC_0: 0.5 x Nominal value$_A$. ≤ Binding$_{MeanA}$ ≤ 2 x Nominal valueA $QC_{Med/High/High_CR}$: Binding$_{MeanA}$ is above Nominal value $- t_{(0.01,df)} * SD_{Nominal\ value}$ or is between $QC_{Low_s/Low_s_CR/Low_i}$ and $QC_{Med/High/High_CR}$[7]: Binding$_{MeanA}$ is within Nominal value $\pm t_{(0.01,df)} * SD_{Nominal\ value}$	0.72 - 2.89 [%B/T]	1.68 - 2.39 [%B/T]	1.73 - 2.50 [%B/T]	1.93 - 2.90 [%B/T]	7.86 - 100.00 or 7.77 - 9.42[7] [%B/T]	47.14 - 100.00 or 46.48 - 57.98[7] [%B/T]	45.38 - 100.00 or 44.46 - 60.61[7] [%B/T]
Tracer robustness[8]	no impact	N/AP	N/AP	N/AP	N/AP	no impact	no impact
Drift (observed difference in %)	-0.7	0.6	N/AP	-6.4	-1.4	0.5	0.6
Assay precision (inter-assay %CV)	7.9	6.0	6.3	6.9	3.3	3.8	5.3
Assay precision (intra-assay %CV)	7.2	4.7	4.7	4.1	3.1	1.7	2.0

[1]Reference item/analyte A was used for all experiments and the marked QC samples. Reference item/analyte B was used only in the sensitivity experiments and the marked QC samples.

[2]T2D serum was used for all experiments except spiking of QC_{Med}, QC_{High} and QC_{High_CR} samples in order to save matrix.

[3]Experiments supporting the choice of MRD were done during method development and are included in the method development report

[4]Initial experiment was repeated per Amendment 2 to the Study Plan (of the study 301510). Reported data comes from the second experiment. Details about both experiments are listed in section 5.7 and Table 45 to Table 50 (of the study 301510).

[5]Haemolysis and lipaemic tests were performed during method development phase and per agreement with Sponsor were not repeated in the validation if no issues were detected. Data regarding haemolysis and lipaemic is included in the method development report or is stored on file at Celerion.

[6]Initial stability testing was successfully performed during method development. Presented data comes from method validation experiments (sample results for low and medium spike were corrected using negative QC samples, see event resolution section 7.2 and Table 51 to Table 62 (of the study 301510). Long term stability was not performed per Sponsor request.

[7]Additional acceptance parameters for $QC_{Med/High/High_CR}$ were calculated only for information purposes (section 5.10 of the study 301510).

[8]Tracer was changed between cut-point runs and used QC samples were within defined acceptance criteria.

表 2.7.1.1-3　抗インスリン イコデク結合抗体のラジオイムノアッセイのカットポイント設定を伴う追加バリデーションで得られた主な結果（Module 5.3.1.4、study 321724）

Validation parameters

Confirmatory cut point (Inhibition cut point, iCP)

Targeted false positive	Calculation	Result
1 %	Robust Parametric Approach: median (%Inhibition) + 2.326 x MADn (%Inhibition)	20.03 %

QC acceptance criteria

QC	Acceptance for %B/T of uninhibited QC (All assay formats)	%Inhibition of inhibited QCs (Confirmatory)	Calculated nominal value of uninhibited QC [%B/T]	Calculated acceptable range of uninhibited QC [%B/T]
QC_0	< NSCP	N/AP	N/AP	N/AP
QC_{Low_i}	≥ NSCP	≥ iCP	N/AP	N/AP
QC_{Med}	≥ Nominal value+ t0.005,df x SD (t0.005, df assumes a one-sided 0.5% failure rate)	≥ iCP	7.16	≥ 6.45
QC_{High}	≥ Nominal value+ t0.005,df x SD (t0.005, df assumes a one-sided 0.5% failure rate)	≥ iCP	43.49	≥ 39.85

Cross-matrices titre precision

The test was performed in 2 matrices: T2D insulin-naïve human serum and healthy human serum.

Six dilution curves were prepared in each matrix. A dilution curve consists of the blank matrix spiked with the purified pAb Rabbit 2854B antibody at 1.00, 2.00, 4.00, 8.00, 16.00, 32.00, 64.00, 128.00, 256.00, 512.00 ng/mL.

Three analytical runs were performed. In each run 2 dilution curves of each matrix at each concentration were tested in screening tier.

Parameter for assessment	Indication of no difference between the matrices	Assessment
%Differences of the responses (%B/T) between 2 curves from both matrix types for each dilution point	%Differences of the responses are < 20% at the dilution points spanning the normalised titre cut point	Assessed in 6 pairs of dilution curves. -no differences of the responses between matrices are indicated in 5 out of 6 dilution pairs. -difference of the responses between matrices is indicated in 1 out of 6 dilution pairs.
End point titre Assessed in case %Difference of the responses is indicated.	The same titre or within 2-fold titre difference	Assessed in 1 pair of dilution curves, that shows %Difference > 20 % at one of the dilutions spanning the normalised titre cut point. No difference, as the titre difference is within 2-fold.

4314 試験に使用した差分法に基づく抗インスリン イコデク結合抗体測定法

血清試料中の残存薬物による影響を最小化するため、実試料（及び対照試料）を酢酸とともにインキュベートした（酢酸は抗体と薬物との結合を解離させる）。次に、中和用 Tris 緩衝液中でポリエチレングリコール 6000（PEG-6000）による沈殿操作を行い、血清試料中の抗体を沈殿させた。遠心分離後、沈殿物を緩衝液で再溶解して、過剰量のヒトインスリン又はインスリン イコデクの存在下及び非

存在下で ^{125}I 標識したトレーサーとともにインキュベートした。一晩インキュベートした後、結合している可能性のある抗原とともに免疫ブロブリンを沈殿させた。洗浄後、沈殿物をガンマカウンターで測定し、添加した総放射能に対する比率、すなわち結合した放射能／添加した総放射能の百分率（%B/T）として放射能を表示した。試料の%B/T 値は、非標識インスリン又はインスリン イコデクを含まない試料の沈殿物中の放射能からバックグラウンド試料中の放射能を引くことで算出した。%B/T が高値であるほど試料中に存在するインスリン又はインスリン イコデクに対する抗体量は多いこととなるが、抗体濃度と%B/T 値との間には必ずしも直線性は認められない。2 型糖尿病患者集団におけるインスリン イコデク特異抗体の基準範囲上限は 1.1% B/T（偽陽性率 1%）であり、2 型糖尿病患者集団におけるヒトインスリン抗体の基準範囲上限は 4.4% B/T（偽陽性率 1%）である。本測定法はバリデーション済みである（Module 5.3.1.4、study 212290）。

4314 試験に使用した抗インスリン イコデク抗体の IgE サブタイプ測定法

イムノキャップ法は、キャップを関連する薬物でコーティングした直接 ELISA 法を一部変更した手法である。キャップは、ELISA プレートのウェルよりも結合能の高いセルローススポンジマトリックスを含む反応チャンバーからなる。ビオチン化インスリン イコデクを、ストレプトアビジンでコートされたキャップとインキュベートした。試料とのインキュベート後、キャップを洗浄して抗ヒト IgE 抗体（ガラクトシダーゼ標識）を添加した。洗浄後、反応液とともにインキュベートし、反応停止液を添加してキャップのシグナル強度を測定した。総 IgE の検量線から、測定用試料及び QC 試料のシグナル強度を 1 リットルあたりのキロ単位のアレルゲン特異的抗体（kUA/L）に変換した。定量下限値は 0.1 であった。本測定法はバリデーション済みである（Module 5.3.1.4、study 215039）。

4314 試験に使用した抗インスリン イコデク抗体の IgG4 サブタイプ測定法

IgG4 測定には、捕捉抗体として抗インスリン イコデク抗体（NN454-1 F31 mAb）を ELISA プレートに一晩コーティングした後にインスリン イコデクを添加するサンドイッチ ELISA 法を用いた。20 倍希釈した前処理済みの QC 試料及び実試料を、試料 A（阻害なし）及び試料 B（過剰量のインスリン イコデクによる阻害あり）として添加した。試料の前処理法は、血清試料中に存在する免疫複合体を解離させるための酸処理と、それに続く Tris 緩衝液による中和及び PEG による沈殿処理であった。沈殿物を溶解させ、試料を ELISA プレートへ移した。インキュベーション及び洗浄を行った後に、抗ヒト IgG4 抗体（HRP 標識）を添加した。TMB に反応させた後に、酸を添加して反応を停止させ、吸光度を測定した。本測定法では、バックグラウンド値で補正した OD 値として結果を示す（吸光度 A－吸光度 B）。本測定法のバリデーション試験において、偽陽性率 1%とした場合の試料の陽性／陰性を判定するための固定カットポイントの算出により、0.1637 に決定した（Module 5.3.1.4、study 301493）。

以上より、すべての測定法に対して現行のガイドライン[3, 4]に準拠したフルバリデーションが実施され、いずれの測定法もバリデーションパラメータのすべての要件に適合した。

2.7.1.2　個々の試験結果の要約

1 つの試験（4572 試験）において、2 型糖尿病患者を対象として、異なる投与部位（大腿部、腹部及び上腕部）へのインスリン イコデク単回投与後のバイオアベイラビリティを検討した。本試験のデザイン、結果及び結論の詳細な記述については、4572 試験の治験総括報告書（Module 5.3.1.1）に示す。

本試験は、2 型糖尿病患者を対象として異なる投与部位に投与後のインスリン イコデクの薬物動態及び薬力学的作用特性を検討する無作為割り付け、1 施設、非盲検、3 期クロスオーバー試験であった。計 24 例の被験者を無作為に割り付ける計画であった。3 回の投与来院において 5.6 単位/kg/週のインスリン イコデクの単回皮下投与（腹部、上腕部及び大腿部）を 3 回行う、6 つの投与順序のいずれか 1 つに被験者を均等に無作為に割り付けた。

2 型糖尿病患者を対象として、大腿部、腹部及び上腕部にインスリン イコデクを投与した時の単回皮下投与後及び予測した定常状態における薬物動態特性を比較した結論を以下に示す。

- 単回投与後の総曝露量（$AUC_{Ico,0\text{-}inf,SD}$）は投与部位間で同程度であった（表 2.7.1.2-1）
- 単回投与後及び予測した定常状態における最高濃度（$C_{max,Ico,SD}$ 及び $C_{max,Ico,SS,model}$）は、大腿部と比較して腹部及び上腕部で高かった（表 2.7.1.2-1）
- 単回投与後の最高濃度到達時間（$t_{max,Ico,SD}$）の中央値は、大腿部で 27 時間、腹部及び上腕部で 24 時間であった
- モデルから予測した定常状態における最高濃度到達時間（$t_{max,Ico,SS,model}$）の中央値は、大腿部で 20 時間、腹部で 17 時間、上腕部で 16 時間であった
- 単回投与後の半減期（$t_{1/2,Ico,SD}$）の調和平均は、大腿部で 149 時間、腹部及び上腕部で 148 時間であった

表 2.7.1.2-1　　曝露量比及び濃度比の要約（4572 試験）

Parameter	Injection Region	EIRR	95% CI	P value
Total exposure after single dose ($AUC_{Ico,0\text{-}inf,SD}$)	Abdomen/thigh	1.02	0.96; 1.09	0.4729
	Upper arm/thigh	1.04	0.98; 1.10	0.1621
	Abdomen/upper arm	0.98	0.93; 1.05	0.6103
Maximum concentrations after single dose ($C_{max,Ico,SD}$)	Abdomen/thigh	1.17	1.07; 1.29	0.0016
	Upper arm/thigh	1.24	1.14; 1.35	<0.0001
	Abdomen/upper arm	0.94	0.86; 1.03	0.2051
Maximum concentrations for predicted steady state ($C_{max,Ico,SS,model}$)	Abdomen/thigh	1.11	1.03; 1.19	0.0035
	Upper arm/thigh	1.16	1.10; 1.23	<0.0001
	Abdomen/upper arm	0.95	0.89; 1.02	0.2439

Abbreviations: EIRR Estimated injection region ratios; CI Confidence interval; SD single dose; AUC Area under curve; SS steady state

2 型糖尿病患者を対象として、大腿部、腹部及び上腕部へ単回皮下投与後 36～60 時間における、インスリン イコデクの部分的な血糖降下作用を探索的に検討した薬力学的作用の結論を以下に示す。

- インスリン イコデクの血糖降下作用は、大腿部、腹部及び上腕部への皮下投与後において同様であった。

2 型糖尿病患者を対象として、大腿部、腹部及び上腕部にインスリン イコデクを投与（各投与期間ごとに週 1 回投与を 1 回）した無作為割り付け、1 施設、非盲検、3 期クロスオーバー試験では、インスリン イコデクに関連する新たな安全性の懸念は認められなかった。重篤な有害事象又は重度の有害事象は報告されなかった。重大な低血糖（レベル 3）は報告されなかった。有害事象及び低血糖の分布について、3 つの投与部位（大腿部、腹部及び上腕部）間で特定の傾向や安全性に関する懸念は認められなかった。

2.7.1.3 全試験を通しての結果の比較と解析

本承認申請には、バイオアベイラビリティを検討した試験は 1 試験のみが含まれた。したがって、全試験を通しての結果の比較は本概要において該当しない。

参考文献

1. Food and Drug Administration. Food and Drug Administration: Code of Federal Regulations, 21 CFR Part 320, Bioavailability and Bioequivalence Requirements. 4/1/2012 2012.
2. European Medicines Agency. Note for Guidance on the Investigation of Bioavailability and Bioequivalence (CPMP/EWP/QWP/1401/98). 1998.
3. European Medicines Agency. Guideline on Bioanalytical Method Validation (EMEA/CHMP/EWP/192217/2009 rev. 1 Corr. 2). 21 Jul 2011.
4. Food and Drug Administration, CDER. Guidance for Industry. Bioanalytical Method Validation. May 2018.

インスリン イコデク（遺伝子組換え）

2.7.1.4. 付録
1. 臨床試験の生体試料中薬物濃度分析報告書及びバリデーション報告書の一覧

ノボ ノルディスク ファーマ株式会社

Common Technical Document Summaries

Insulin icodec

2.7.1 Summary of biopharmaceutic studies and associated analytical methods

Appendix 4.1

List of Bioanalytical Reports and Assay Validation Reports in Clinical Trials

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Table 4.1.1 Bioanalytical Method Life Cycle Information – method validations

	Method validation #212348	Method validation #212408	Method validation #216306	Method validation #300072
Analyte	NNC0148-0287	NNC0148-0287	NNC0148-0287	NNC0148-0287
Validation type	Full validation	Partial validation	Full validation	Partial validation
eCTD reference number	Not applicable	Not applicable	Not applicable	Not applicable
Method ID	Method A	Method A	Method B	Method B
Duration of time method in use	09/2012 – 05/2014	10/2012 – 05/2015	01/2017 - 03/2018	03/2017 - 12/2017
Bioanalytical site	Novo Nordisk Park, DK-2760, Måløv. Test site: Development Bioanalysis	Novo Nordisk Park, DK-2760, Måløv. Test site: Development Bioanalysis	Novo Nordisk Park, DK2760 Måløv, Denmark Test Site: Non-clinical Development	Novo Nordisk Park, DK2760 Måløv, Denmark Test Site: Non-clinical Development
Matrix	human serum			
Platform	LOCI			
Format	A validated LOCI immunoassay using acceptor beads conjugated with monoclonal antibody (HUI-018) recognizing human insulin and donor beads with streptavidin binding to biotinylated monoclonal antibody (M-GIIone-2F4A80) directed against the B25H substitution in insulin icodec.			
Stock reference, lot number, expiration date	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM0412 Expiry date: 01-May-2015	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM0412 Expiry date: 01-May-2015	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM0412 Expiry date: 01-May-2020	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM0412 Expiry date: 01-May-2020
Calibration range from LLOQ to ULOQ	200 - 50000 pmol/L	200 - 50000 pmol/L	500 - 150000 pmol/L 500 - 100000 pmol/L	500 - 150000 pmol/L 500 - 100000 pmol/L
Matrix study population	Normal, T1D, T2D	Normal	Normal, T1D, T2D	Normal
Link to reports and applicable amendments	M 5.3.1.4, study 212348	M 5.3.1.4, study 212408	M 5.3.1.4, study 216306	M 5.3.1.4, study 300072
Synopsis of amendment history	Not applicable	Not applicable	Not applicable	Not applicable

	Method validation #306660-CA25310-01	**Method validation #306660-CA25310-02**	**Method validation #322147**	**Method validation #321497**
Analyte	NNC0148-0287	NNC0148-0287	NNC0148-0287	NNC0148-0287
Validation type	Full validation	Partial validation including cross-validation	Partial validation including cross-validation	Full validation including cross-validation
eCTD reference number	Not applicable	Not applicable	Not applicable	Not applicable
Method ID	Method C	Method C	Method C	Method C
Duration of time method is in use	09/2018 - 07/2019	02/2019 - 01/2022	09/2022 – 11/2022	12/2020- 11/2022
Bioanalytical site	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland	Wuxi Apptec Co., Bioanalytical Services, Shanghai, China
Matrix	human serum			
Platform	LOCI			
Format	A validated LOCI immunoassay using acceptor beads conjugated with monoclonal antibody (HUI-018) recognizing human insulin and donor beads with streptavidin binding to biotinylated monoclonal antibody (M-GIIone-2F4A80) directed against the B25H substitution in insulin icodec.			
Stock reference, lot number, expiration date	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM0412 Expiry date: 01-May-2020	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM0412 Expiry date: 01-May-2020 Lot: 1436-PRM18 Expiry date: 21-Nov-2021	Reference drug: NNC0148-0000-0287 Lot: ICO_SRM20 Expiry date: 01-Feb-2023	Reference drug: NNC0148-0000-0287) Lot: 1436_PRM18 Expiry date: 27-May-2024
Calibration range from LLOQ to ULOQ	500 - 80000 pmol/L	500 - 80000 pmol/L	500 - 80000 pmol/L	500 - 80000 pmol/L
Matrix study population	Normal, T1D and T2D	Normal	Renal impaired serum	Normal, Chinese, T2D
Link to reports and applicable amendments	M 5.3.1.4, study 306660-CA25310-01	M 5.3.1.4, study 306660-CA25310-02	M 5.3.1.4, study 322147	M 5.3.1.4, study 321497
Synopsis of amendment history	Not applicable	Not applicable	Not applicable	Not applicable

	Method validation #306661-CA25312-01	**Method validation #306661-CA25312-02**
Analyte	NNC0148-0287	NNC0148-0287
Validation type	Full validation	Partial validation
eCTD reference number	Not applicable	Not applicable
Method ID	Method D	Method D
Duration of time method is in use	10/2018 - 07/2019	02/2019 – 06/2020
Bioanalytical site	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland
Matrix	human urine	
Platform	LOCI	
Format	A validated LOCI immunoassay using acceptor beads conjugated with monoclonal antibody (HUI-018) recognizing human insulin and donor beads with streptavidin binding to biotinylated monoclonal antibody (M-GIIone-2F4A80) directed against the B25H substitution in insulin icodec.	
Stock reference, lot number, expiration date	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM0412 Expiry date: 01-May-2020	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM0412 Expiry date: 01-May-2020
Calibration range from LLOQ to ULOQ	150 - 10000 pmol/L	150 - 10000 pmol/L
Matrix study population	Normal	Normal
Link to reports and applicable amendments	M 5.3.1.4, study 306661-CA25312-01	M 5.3.1.4, study 306661-CA25312-02
Synopsis of amendment history	Not applicable	Not applicable

Table 4.1.2 Bioanalytical Method Life Cycle Information – clinical studies

	Clinical study #1 (4314)	Clinical study #2 (4226)	Clinical study #3 (4225)	Clinical study #4 (4422)
Analyte	NNC0148-0000-0287	NNC0148-0000-0287	NNC0148-0000-0287	NNC0148-0000-0287
Validation type	In-study	In-study	In-study	In-study
eCTD reference number	Not applicable	Not applicable	Not applicable	Not applicable
Method ID	Method A	Method D	Method C	Method C
Duration of time method is in use	02/2017 – 04/2018	03/2019 – 01/2020	04-2019 - 10/2020	05/2019 – 03/2020
Bioanalytical site	Non-clinical Development, Novo Nordisk Park, DK2760 Måløv, Denmark Test Site: Non-clinical Development	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland
Matrix	Human serum	Human urine	Human serum	
Platform	LOCI			
Format	A validated LOCI immunoassay using acceptor beads conjugated with monoclonal antibody (HUI-018) recognizing human insulin and donor beads with streptavidin binding to biotinylated monoclonal antibody (M-GIIone-2F4A80) directed against the B25H substitution in insulin icodec.			
Stock reference, lot number, expiration date	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM0412 Expiry date: 01-May 2017 and 01-May 2020	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM0412 Expiry date: 01-May 2020	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM0412 and 1436_PRM18 Expiry date: 01-May 2020 and 27-May-2024	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM0412 and 1436_PRM18 Expiry date: 01-May 2020
Calibration range from LLOQ to ULOQ	A: 500 - 150000 pmol/L B: 500 - 100000 pmol/L	150 - 10000 pmol/L	500 – 80000 pmol/L	500 – 80000 pmol/L
Matrix study population	T2D	Renal impaired subjects	T1D	T1D, Japanese
Link to reports and applicable amendments	M 5.3.4.2, Trial 4314, Appendix 16.2.8, Bioanalytical report, Novo Nordisk, study 300039	M 5.3.3.3, Trial 4226, Appendix 16.2.9, study CA26323-02	M 5.3.1.4, study 306660-CA25310-01 and study 306660-CA25310-02	M 5.3.1.4, study 306660-CA25310-01 and study 306660-CA25310-02
Synopsis of amendment history	Not applicable	Not applicable	Not applicable	Not applicable

	Clinical study #5 (4462)	**Clinical study #6 (4569)**	**Clinical study #7 (4570)**	**Clinical study #8 (4571)**
Analyte	NNC0148-0000-0287	NNC0148-0000-0287	NNC0148-0000-0287	NNC0148-0000-0287
Validation type	In-study	In-study	In-study	In-study
eCTD reference number	Not applicable	Not applicable	Not applicable	Not applicable
Method ID	Method C	Method C	Method C	Method C
Duration of time method is in use	10/2019 – 05/2022	02/2021 - 08/2022	01/2021 – 07/2022	10/2021 - 09/2022
Bioanalytical site	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland	Wuxi Apptec Co., Bioanalytical Services, Shanghai, China
Matrix	Human serum			
Platform	LOCI			
Format	A validated LOCI immunoassay using acceptor beads conjugated with monoclonal antibody (HUI-018) recognizing human insulin and donor beads with streptavidin binding to biotinylated monoclonal antibody (M-GIIone-2F4A80) directed against the B25H substitution in insulin icodec			
Stock reference, lot number, expiration date	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM0412 and 1436_PRM18 Expiry date: 01-May-2020, 21-Nov-2021	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM18 Expiry date: 21-Nov-2021 and 21-Jun-2023	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM18 Expiry date: 21-Nov-2021 and 21-Jun-2023	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM18 Expiry date: 27-May-2024
Calibration range from LLOQ to ULOQ	500 – 80000 pmol/L	500 – 80000 pmol/L	500 – 80000 pmol/L	500 – 80000 pmol/L
Matrix study population	T2D	T2D	Hepatic impaired subjects	T2D, Chinese
Link to reports and applicable amendments	M 5.3.1.4, study 306660-CA25310-01 and study 306660-CA25310-02	M 5.3.4.2, Trial 4569, Appendix 16.2.9, study CA32864-01	Selectivity in hepatic impairment serum In-study; M 5.3.3.3, Trial 4570, Appendix 16.2.9, study CA32983	M 5.3.3.3, Trial 4571, Appendix 16.2.9, study 407279-214227-PSA-HGR
Synopsis of amendment history	Not applicable	Not applicable	Not applicable	Not applicable

	Clinical study #9 (4572)	**Clinical study #10 (4383)**	**Clinical study #11 (4478)**	**Clinical study #12 (4479 China)**
Analyte	NNC0148-0000-0287	NNC0148-0000-0287	NNC0148-0000-0287	NNC0148-0000-0287
Validation type	In-study	In-study	In-study	In-study
eCTD reference number	Not applicable	Not applicable	Not applicable	Not applicable
Method ID	Method C	Method C	Method C	Method C
Duration of time method is in use	04/2021 – 03/2022	03/2019 – 04/2020	06/2021 – 05/2022	03/2022 - 09/2022
Bioanalytical site	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland	Wuxi Apptec Co., Bioanalytical Services, Shanghai, China
Matrix	Human serum			
Platform	LOCI			
Format	A validated LOCI immunoassay using acceptor beads conjugated with monoclonal antibody (HUI-018) recognizing human insulin and donor beads with streptavidin binding to biotinylated monoclonal antibody (M-GIIone-2F4A80) directed against the B25H substitution in insulin icodec			
Stock reference, lot number, expiration date	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM18 Expiry date: 21-Nov-2021	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM0412 Expiry date: 01-May-2020	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM18 Expiry date: 21-Nov-2021 and 21-Jun-2023	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM18 Expiry date: 27-May-2024
Calibration range from LLOQ to ULOQ	500 – 80000 pmol/L	500 – 80000 pmol/L	500 – 80000 pmol/L	500 – 80000 pmol/L
Matrix study population	T2D	T2D	T2D	T2D
Link to reports and applicable amendments	M 5.3.1.1, Trial 4572, Appendix 16.2.9, study CA32867-01	M 5.3.5.1, Trial 4383, Appendix 16.2.9, study CA25157-01	M 5.3.5.1, Trial 4478, Bioanalytical Report, study CA31565-01	M 5.3.5.1, Trial 4479, Bioanalytical Report - Wuxi, study 407279-214197-PSA-HGR
Synopsis of amendment history	Not applicable	Not applicable	Not applicable	Not applicable

	Clinical study #13 (4480)	**Clinical study #14 (4625)**	**Clinical study #15 (4479 ROW)**	**Clinical study #16 (4226)**
Analyte	NNC0148-0000-0287	NNC0148-0000-0287	NNC0148-0000-0287	NNC0148-0000-0287
Validation type	In-study	In-study	In-study	In-study
eCTD reference number	Not applicable	Not applicable	Not applicable	Not applicable
Method ID	Method C	Method C	Method C	Method C
Duration of time method is in use	02/2022 – 10/2022	12/21 - 08/2022	01/2022 – 10/2022	03/2019 - 01/2020
Bioanalytical site	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland	Celerion, Allmendstrasse 32, 8320 Fehraltorf, Switzerland
Matrix	Human serum			
Platform	LOCI			
Format	A validated LOCI immunoassay using acceptor beads conjugated with monoclonal antibody (HUI-018) recognizing human insulin and donor beads with streptavidin binding to biotinylated monoclonal antibody (M-GIIone-2F4A80) directed against the B25H substitution in insulin icodec			
Stock reference, lot number, expiration date	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM18 Expiry date: 21-Nov-2024 and 21-Jun-2023	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM18 Expiry date: 21-Jun-2023	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM18 Expiry date: 21-Nov-2021 and 21-Jun-2023	Reference drug: NNC0148-0000-0287 Lot: 1436_PRM0412 Expiry date: 01-May-2020
Calibration range from LLOQ to ULOQ	500 – 80000 pmol/L	500 – 80000 pmol/L	500 – 80000 pmol/L	500 – 80000 pmol/L
Matrix study population	T2D	T1D	T2D	renal impairment subjects
Link to reports and applicable amendments	M 5.3.5.1, Trial 4480, Bioanalytical Report, study CA31552-01	M 5.3.5.1, Trial 4625, Bioanalytical Report, study CA33523-01	M 5.3.5.1, Trial 4479, Bioanalytical Report - Celerion, study CA31566-01	M 5.3.3.3, Trial 4226, Appendix 16.2.9, study CA26323-01
Synopsis of amendment history	Not applicable	Not applicable	Not applicable	Not applicable

Table 4.1.3 Summary Method Performance

Bioanalytical method validation report name, amendments, and hyperlink	**Validation of a New Analytical Range and Cross-reactivity of Insulin Degludec in the Insulin 287 LOCI in Human Serum (M 5.3.1.4, study 216306), including reference to M 5.3.1.4, study 212348, M 5.3.1.4, study 212408 and M 5.3.4.2, Trial 4314, Appendix 16.2.8, study 300039**
Method description	The assay is a homogenous Luminescence Oxygen Channeling Immunoassay (LOCI), based on the proximity of two latex bead reagents; donor and acceptor beads. The donor beads are coated with streptavidin and binds the biotinylated antibody (M-GIIone-2F4A80), specific for the B25H mutation in insulin icodec. The acceptor beads are conjugated with the monoclonal antibody (HUI-018), recognizing human insulin. The three reactants are brought in close proximity in the presence of insulin icodec and form a bead-aggregate immunocomplex. Excitation of a photosensitizer present in the donor beads converts ambient oxygen to singlet oxygen which channels into the acceptor beads and triggers chemiluminescence that is measured in the Envision plate reader. In study 216306, the analytical range from study 212348 was optimized. Originally, a range from 500-150000 pmol/L was validated (range A) but this range was truncated to 500 – 100000 pmol/L (range B) to increase assay robustness. Unless otherwise stated, the listed parameters refer to analytical range B.
Materials used for standard calibration curve and concentration	Pool of human serum (mixed gender) spiked with insulin icodec Analytical range A. 0[a], 250*, 500, 1000, 3000, 10000, 25000, 50000, 100000, 125000, and 150000 pmol/L Analytical range B. 0[a], 250*, 500, 1000, 3000, 10000, 25000, 50000, 80000, 100000 and 120000[a] pmol/L ([a]anchor calibrator point)
Validated assay range	Analytical range A; 500 – 150000 pmol/L Analytical range B; 500 – 100000 pmol/L
Material used for Quality controls (QCs) and concentration	Pool of human serum (mixed gender) spiked with insulin icodec Analytical range A. LLOQ-QC:500, low-QC: 1500, medium-QC:12000, high-QC:120000 and ULOQ: 150000 (all in pmol/L) Analytical range B. LLOQ-QC:500, low-QC:1500, medium-QC:12000, high-QC:75000 and ULOQ:100000 (all in pmol/L)
Minimum required dilution (MRDs)	50 times (5x dilution in human serum and 10x dilution in LOCI buffer before analysis)
Source and lot of reagents	Biotinylated antibody M-GIIone-2F4A80 Supplier: Novo Nordisk Batch: 05-Jan-2017/TiAd HUI-018 conjugated acceptor beads Supplier: Novo Nordisk Batch: 20-Sep-2016/TiAd Streptavidin coated donor beads Supplier: Perkin Elmer Batch: 180375900, 189323630, 193128226
Regression model and weighting	5 parametric logistic (5-PL) regression with a $1/Y^2$ weighting factor

Validation parameters	Method validation summary		Source location
Standard calibration curve performance during accuracy and precision runs	Number of calibrators from LLOQ to ULOQ	range A: 9 range B: 8	M 5.3.1.4, study 216306, Section 2.2
	Cumulative accuracy (%bias) from LLOQ to ULOQ	range A: -6.7% to 7.0% range B: -1.8% to 6.4%	M 5.3.1.4, study 216306, Table 22 and Table 23
	Cumulative precision (%CV) from LLOQ to ULOQ	range A: ≤7.2% range B: ≤6.5%	
Performance of QCs during precision and accuracy runs	**Cumulative accuracy (%bias) in 5 QCs**	range A: -12.6% to 0.0% range B: -12.2% to 0.0%	M 5.3.1.4, study 216306, Table 7 and Table 14 Table 8 and Table 15
	Inter-batch %CV	range A: ≤14.6% range B: ≤9.7%	
	Total error (TE)	range A: ≤19.7% range B: ≤20.0%	
Selectivity & matrix effect	Selectivity was performed in human serum at 0, 1200 (LLOQ) and 120000 (high-QC) pmol/L in three different populations with 10 individual samples in each population.		M 5.3.1.4, study 216306, Table 3
	Healthy serum samples 8 of 10 passed: -6.7% to 16.7%bias 2 subjects failing at 120000 pmol/L: -22.6% and 27.5%bias		M 5.3.1.4, study 216306, Table 17
	T1D serum samples 10 out of 10 passed: -17.6 to 24.2%bias		M 5.3.1.4, study 216306, Table 18
	T2D serum samples 9 out of 10 passed: -16.9% to 15.4%bias 1 subject failing at 120000 pmol/L: -23.3%bias		M 5.3.1.4, study 216306, Table 19
	Selectivity at LLOQ was validated in three other validation studies		M 5.3.1.4, study 306660-CA25310-01 and study 321497
Interference & specificity	Specificity of the assay was tested with the following compounds: human insulin, insulin aspart, insulin detemir and insulin glargine at 5000 pmol/L level of insulin icodec		M 5.3.1.4, study 212348, Table 9
	insulin Degludec at LLOQ and ULOQ levels of insulin icodec		M 5.3.1.4, study 212348, Table 20

	Acceptance criteria were met for each concentration of each analogue All analogues were tested again at LLOQ and ULOQ levels in two other validation studies	M 5.3.1.4, study 306660-CA25310-01 and study 321497
Hemolysis effect	Serum with no and 4 different levels of haemolytic grade (0-4) was tested. All samples met acceptance criteria: -9.25% to-1.58%bias	M 5.3.1.4, study 212348, Table 10 and Table 26
Lipemic effect	Not applicable	Not applicable
Dilution linearity & hook effect	Dilution linearity: Highest concentration tested at ULOQ (150000 pmol/L, range A) in dilutions: undiluted, 1/5, 1/20, 1/100, All samples met acceptance criteria: -20.0% to -12.0%bias Hook effect: No hook effect was observed up to 5000000 pmol/L	M 5.3.1.4, study 216306, Table 9 and Table 16 M 5.3.1.4, study 212348, Table 8, Table 24 and Table 25
Bench-top/process stability	Serum: 24 hours at RT Whole blood: Up to 1h on ice and at RT	M 5.3.1.4, study 212348, Table 12 and Table 28 M 5.3.1.4, study 212348, Table 13
Freeze-thaw stability	5 freeze-thaw cycles at -20°C	M 5.3.1.4, study 212348, Table 11 and Table 27
Long-term stability	Up to 17 months at -20°C	M 5.3.1.4, study 212408, Table 3 and Appendix D
Parallelism	Parallelism investigated in-study, trial 4314: 6 samples near C_{max} tested, %CV ≤ 10.0% across dilutions and across samples	M 5.3.4.2, Trial 4314, Appendix 16.2.8, study 300039, Appendix G
Carry over	Not applicable	Not applicable
Method performance in study (4314) Study 300039 Bioanalysis NNC0148-0000-0287 in Trial ID: NN1436-4314		
Assay passing rate	A total of 114 runs out of 117 (97.4%) were accepted according to the run acceptance criteria.	M 5.3.4.2, Trial 4314, Appendix 16.2.8, study 300039, Table 3
Standard curve performance	• Cumulative bias: range A: -7.3% to 4.8% range B: -1.2% to 4.6% • Cumulative precision: range A: ≤8.1% range B: ≤8.1%	M 5.3.4.2, Trial 4314, Appendix 16.2.8, study 300039, Appendix B
QC performance	• Cumulative bias range: range A: -4.2% to -2.5% range B: -4.2% to 2.4%	M 5.3.4.2, Trial 4314, Appendix 16.2.8, study 300039, Appendix A

	• Cumulative precision: range A: ≤14.1% range B: ≤10.5% • TE: not applicable	
Method reproducibility	Incurred sample re-analysis was performed in 206/2552 (8.1%) of study samples, and 98.1% of the samples met acceptance criteria	M 5.3.4.2, Trial 4314, Appendix 16.2.8, study 300039, Appendix F
Study sample analysis/stability	Study samples, calibrators, and QC's were all analysed within the validated long-term stability for 17 months at -20C	M 5.3.4.2, Trial 4314, Appendix 16.2.8, study 300039, Section 2.4 M 5.3.1.4, study 212408, Table 3 and Appendix D
Standard calibration curve performance during accuracy and precision runs	Analytical range A. 0*, 250*, 500, 1000, 3000, 10000, 25000, 50000, 100000, 125000, 150000 pmol/L Analytical range B. 0*, 250*, 500, 1000, 3000, 10000, 25000, 50000, 80000, 100000, 120000* pmol/L *anchor points	

Bioanalytical method validation report name, amendments, and hyperlink	**Validation of an AlphaLisa method for the quantitative determination of insulin 0287 in human serum (M 5.3.1.4, study 306660-CA25310-01) and Partial validation of an AlphaLisa method for the quantitative determination of insulin 0287 in human serum – Extension of long term stability, method automation, cross-reactivity with insulin degludec and cross-validation with sponsor supplied QC samples (M 5.3.1.4, study 306660-CA25310-02)**
Method description	The LOCI method was originally developed and validated at Novo Nordisk (M 5.3.1.4, study 212348 and study 216306). Prior to initiation of phase 2 clinical trials, the assay was transferred to Celerion, Zürich, Switzerland and validated in human serum in study 306660-CA25310-01, and the analytical range was truncated from 500-100000 pmol/L to 500-80000 pmol/L to ensure robustness of the assay across different batches of donor and acceptor beads. Extension of long-term stability, method automation, cross-reactivity with insulin degludec and cross-validation with sponsor supplied QC samples was validated in study 306660-CA25310-02. VCA25310-01 is the primary validation report whereas long term stability (LTS) (at -20°C) and cross-validation with sponsor supplied QC samples was assessed in the partial-validation report VCA25310-02. Selectivity in renal impaired serum and cross-reactivity with semaglutide was validated in M 5.3.1.4, study 322147 whereas selectivity in hepatic impaired serum was validated in-study 4570 in report M 5.3.3.3, Trial 4570, Appendix 16.2.9, study CA32983. In addition, dilution quality control samples were included in relevant analytical runs, i.e., analytical runs where diluted samples were applied. The dilution quality controls were diluted in the same manner as the clinical samples for verification of the dilution step.
Materials used for standard calibration curve and concentration	Human serum, 8 concentration levels and 3 anchor points: 0[a,b], 250[b], 500, 1000, 3000, 10000, 20000, 40000, 64000, 80000 and 100000[b] (in pmol/L) [a]blank sample, [b]anchor point
Validated assay range	500 – 80000 pmol/L (changed from 500 - 100000 pmol/L in validation study 216306)
Material used for Quality controls (QCs) and concentration	Human serum LLOQ-QC:500.0, Low-QC:1500, Medium-QC:12000, High-QC:60000, ULOQ:80000, Dilution-QC:2943090 (in pmol/L)
Minimum required dilution (MRDs)	50
Source and lot of reagents	Biotinylated M-GIIone-2F4A80B65 Source: Novo Nordisk A/S Batch: 15-May-2018/TiAd Conjugated acceptor beads with HUI-018 Source: Novo Nordisk A/S Batch: HUI-018-270718/TiAd Streptavidin-coated donor beads Source: Perkin Elmer Batch: 2298681
Regression model and weighting	5-parametric logistic (5-PL) regression with a $1/Y^2$ weighting factor

Validation parameters	Method validation summary		Source location
Standard calibration curve performance during accuracy and precision runs	Number of calibrators from LLOQ to ULOQ	8	M 5.3.1.4, study 306660-CA25310-01, Table 2
	Cumulative accuracy (%bias) from LLOQ to ULOQ	-2.9% to 6.3%	
	Cumulative precision (%CV) from LLOQ to ULOQ	≤5.8%	
Performance of QCs during precision and accuracy runs	**Cumulative accuracy (%bias) in 5 QCs**	-12.0% to -3.6%	M 5.3.1.4, study 306660-CA25310-01, Table 8
	Inter-batch %CV	≤18.2%	
	Total error (TE)	≤29.9%	
Selectivity & matrix effect	Selectivity was performed in human serum at 0, 500 (LLOQ) and 60000 pmol/L (high-QC) in three different populations with 10 individual samples in each population: Non-diabetic healthy individuals including two lipemic samples: 8 out of 10 passed: 84.4% to 116.0%bias 2 out of 10 failed at high-QC: 120.2% and 123.2%bias T1D individuals: 8 out of 10 passed: 92.6% to 119.5%Bias 2 out of 10 failed at high-QC: 121.2% and 126.3%bias T2D individuals (repeated run 54): 10 out of 10 passed: 94.7% to 125.0%bias		M 5.3.1.4, study 306660-CA25310-01, Section 5.4 M 5.3.1.4, study 322147, Section 6.3 M 5.3.3.3, Trial 4570, Appendix 16.2.9, study CA32983, Section 6.7 M 5.3.1.4, study 306660-CA25310-01, Table 10
Interference & specificity	Specificity was tested with the following compounds: human insulin, insulin aspart, insulin detemir and insulin glargine at LLOQ and ULOQ levels of insulin icodec higher concentration of insulin degludec at LLOQ and ULOQ levels of insulin icodec Acceptance criteria were met for each concentration of each analogue		M 5.3.1.4, study 306660-CA25310-01, Table 12
Hemolysis effect	3 lots tested with 3% hemolysis degree, all lots met acceptance criteria: 99.3% to 113.2%bias		M 5.3.1.4, study 306660-CA25310-01, Table 11
Lipemic effect	2 lots tested, all lots met acceptance criteria: 84.4% to 113.5%bias		M 5.3.1.4, study 306660-CA25310-01, Table 10
Dilution linearity & hook effect	Dilution linearity: Highest concentration tested at 2943090 pmol/L in dilutions: undiluted, 1/10, 1/50, 1/100 and 1/300		M 5.3.1.4, study 306660-CA25310-01, Table 13

	The undiluted sample and dilution 1/10 were above ULOQ. All other dilutions met acceptance criteria: -18.5% to 19.2%bias Hook effect: No high dose hook effect was observed up to 2943090 pmol/L	
Bench-top/process stability	Human serum 22h at RT Whole blood 2h at 5^{0}C and RT	M 5.3.1.4, study 306660-CA25310-01, Table 14 M 5.3.1.4, study 306660-CA25310-01, Table 16
Freeze-thaw stability	6 freeze-thaw cycles at -20°C	M 5.3.1.4, study 306660-CA25310-01, Table 14
Long-term stability	34 days at -20°C and at -80°C 733 days at -20°C	M 5.3.1.4, study 306660-CA25310-01, Table 15 M 5.3.1.4, study 306660-CA25310-02, Table 8
Parallelism	Parallelism investigated in-study, trial **4569**: - 6 samples below ULOQ to be able to include undiluted samples, %CV ≤ 7.2% across dilutions and across samples - 6 samples near C_{max}, %CV ≤ 18.5% across dilutions and across samples	M 5.3.4.2, Trial 4569, Appendix 16.2.9, study CA32864-01, Table 15
Carry over	Not applicable	Not applicable
Method performance in study (4226) Determination of insulin 287 in human serum samples from "Investigation of Pharmacokinetics and Safety Profile of a Single Dose NNC0148-0287 C (insulin 287) in Subjects with Various Degrees of Renal Impairment" by an AlphaLisa method - Study CA26323-01		
Assay passing rate	A total of 85 runs out of 85 (100%) were accepted according to the run acceptance criteria.	M 5.3.3.3, Trial 4226, Appendix 16.2.9, study CA26323-01, Table 1
Standard curve performance	• Cumulative bias range: -2.8% to 4.8% • Cumulative precision: ≤5.0%	M 5.3.3.3, Trial 4226, Appendix 16.2.9, study CA26323-01, Table 2
QC performance	• Cumulative bias range: 0.9% to 6.4% • Cumulative precision: ≤11.4% • TE: not applicable	M 5.3.3.3, Trial 4226, Appendix 16.2.9, study CA26323-01, Table 4
Method reproducibility	Incurred sample re-analysis was performed in 143/1584 (9.0%) of study samples, and 98.6% of the samples met acceptance criteria	M 5.3.3.3, Trial 4226, Appendix 16.2.9, study CA26323-01, Section 5.6 and Table 8
Study sample analysis/stability	Study samples, calibrators and QC's were all analysed within the validated long-term stability for 24 months at -20°C	M 5.3.3.3, Trial 4226, Appendix 16.2.9, study CA26323-01, Section 3.7.2 M 5.3.1.4, 306660-CA25310-02, Table 8

Standard calibration curve performance during accuracy and precision runs	8 concentration levels and 3 anchor points 0[a,b], 250b, 500, 1000, 3000, 10000, 20000, 40000, 64000, 80000 and 100000[b] (in pmol/L) [a]blank sample, [b]anchor point	
Method performance in study (4225) Determination of insulin 287 in human serum samples from "A Trial Investigating the Pharmacokinetics and Pharmacodynamics of NNC0148-0287 C (insulin 287) at Steady State Conditions in Subjects with Type 1 Diabetes" by an AlphaLisa method - Study CA26321-01		
Assay passing rate	A total of 158 runs out of 161 (98.1%) were accepted according to the run acceptance criteria.	M 5.3.4.2, Trial 4225, Appendix 16.2.9, study CA26321-01, Table 1
Standard curve performance	• Cumulative bias range: -2.1% to 2.9% • Cumulative precision: ≤5.2%	M 5.3.4.2, Trial 4225, Appendix 16.2.9, study CA26321-01, Table 2
QC performance	• Cumulative bias range: 0.4% to 6.4% • Cumulative precision: ≤8.6% • TE: 100.1 to 106.4%	M 5.3.4.2, Trial 4225, Appendix 16.2.9, study CA26321-01, Table 6
Method reproducibility	Incurred sample re-analysis was performed in 248/3502 (7.08%) of study samples, and 98.8% of the samples met acceptance criteria	M 5.3.4.2, Trial 4225, Appendix 16.2.9, study CA26321-01, Table 11
Study sample analysis/stability	Study samples, calibrators and QC's were all analysed within the validated long-term stability for 24 months at -20°C	M 5.3.4.2, Trial 4225, Appendix 16.2.9, study CA26321-01, Section 2.7.4 M 5.3.1.4, study 306660-CA25310-02, Table 8
Standard calibration curve performance during accuracy and precision runs	8 concentration levels and 3 anchor points 0[a,b], 250b, 500, 1000, 3000, 10000, 20000, 40000, 64000, 80000 and 100000[b] (in pmol/L) [a]blank sample, [b]anchor point	
Method performance in study (4422) Determination of insulin 287 in human serum samples from "A multiple-dose trial investigating the safety, tolerability, pharmacokinetics and pharmacodynamics of NNC0148-0287 C (insulin 287) for subcutaneous administration in Japanese subjects with type 1 diabetes" by an AlphaLisa method - Study CA26569-01		
Assay passing rate	A total of 56 runs out of 56 (100%) were accepted according to the run acceptance criteria.	M 5.3.4.2, Trial 4422, Appendix 16.2.9, study CA26569-01, Table 1
Standard curve performance	• Cumulative bias range: -3.1% to 2.8% • Cumulative precision: ≤5.2%	M 5.3.4.2, Trial 4422, Appendix 16.2.9, study CA26569-01, Table 2
QC performance	• Cumulative bias range: 0.5% to 8.2% • Cumulative precision: ≤8.6% • TE: not available	M 5.3.4.2, Trial 4422, Appendix 16.2.9, study CA26569-01, Table 4
Method reproducibility	Incurred sample re-analysis was performed in 120/1151 (10.4%) of study samples, and 100% of the samples met the acceptance criteria	M 5.3.4.2, Trial 4422, Appendix 16.2.9, study CA26569-01, Table 8

Study sample analysis/stability	Study samples, calibrators and QC's were all analysed within the validated long-term stability for 24 months at -20°C	M 5.3.4.2, Trial 4422, Appendix 16.2.9, study CA26569-01, Section 3.7.4 M 5.3.1.4, 306660-CA25310-02, Table 8
Standard calibration curve performance during accuracy and precision runs	8 concentration levels and 3 anchor points 0[a,b], 250b, 500, 1000, 3000, 10000, 20000, 40000, 64000, 80000 and 100000[b] (in pmol/L) [a]blank sample, [b]anchor point	
Method performance in study (4462) Determination of insulin 287 in human serum samples from "A Trial Investigating the Hypoglycaemic Response to Overdosing of NNC0148-0287 C (Insulin 287) in Subjects with Type 2 Diabetes" by an AlphaLisa method - Study CA27576-01		
Assay passing rate	A total of 64 runs out of 67 (95.5%) were accepted according to the run acceptance criteria.	M 5.3.4.2, Trial 4462, Appendix 16.2.9, study CA27576-01, Table 2
Standard curve performance	• Cumulative bias range: -2.6% to 4.7% • Cumulative precision: ≤6.1%	
QC performance	• Cumulative bias range: -0.1% to 1.8% • Cumulative precision: ≤8.1%% • TE: not applicable	M 5.3.4.2, Trial 4462, Appendix 16.2.9, study CA27576-01, Table 6
Method reproducibility	Incurred sample re-analysis was performed in 123/1153 (10.7%) of study samples, and 96.8% of the samples met acceptance criteria	M 5.3.4.2, Trial 4462, Appendix 16.2.9, study CA27576-01, Table 11
Study sample analysis/stability	Study samples, calibrators and QC's were all analysed within the validated long-term stability for 24 months at -20°C	M 5.3.4.2, Trial 4462, Appendix 16.2.9, study CA27576-01, Section 4.7.4 M 5.3.1.4, study 306660-CA25310-02, Table 8
Standard calibration curve performance during accuracy and precision runs	8 concentration levels and 3 anchor points 0[a,b], 250b, 500, 1000, 3000, 10000, 20000, 40000, 64000, 80000 and 100000[b] (in pmol/L) [a]blank sample, [b]anchor point	
Method performance in study (4569) Determination of insulin icodec and in-study validation Parallelism- in human serum samples from "A Trial Investigating the Pharmacokinetic and Pharmacodynamic Properties of insulin icodec in Subjects with Type 2 Diabetes" by an AlphaLisa method - Study CA32864-01		
Assay passing rate	A total of 113 runs out of 115 (98.3%) were accepted according to the run acceptance criteria.	M 5.3.4.2, Trial 4569, Appendix 16.2.9, study CA32864-01, Table 1
Standard curve performance	• Cumulative bias range: -4.8 to 8.7% • Cumulative precision: ≤5.7%	M 5.3.4.2, Trial 4569, Appendix 16.2.9, study CA32864-01, Table 2
QC performance	• Cumulative bias range: -2.7 to –0.7% • Cumulative precision: ≤8.2% • TE: not applicable	M 5.3.4.2, Trial 4569, Appendix 16.2.9, study CA32864-01, Table 8

Method reproducibility	Incurred sample re-analysis was performed in 173/2295 (7.5%) of study samples, and 98.8% of the samples met the pre-specified criteria	M 5.3.4.2, Trial 4569, Appendix 16.2.9, study CA32864-01, Table 14
Study sample analysis/stability	Study samples, calibrators and QC's were all analysed within the validated long-term stability for 24 months at -20°C	M 5.3.4.2, Trial 4569, Appendix 16.2.9, study CA32864-01, Section 4.7.4 M 5.3.1.4, study 306660-CA25310-02, Table 8
Standard calibration curve performance during accuracy and precision runs	8 concentration levels and 3 anchor points 0[a,b], 250[b], 500, 1000, 3000, 10000, 20000, 40000, 64000, 80000 and 100000[b] (in pmol/L) [a]blank sample, [b]anchor point	
Method performance in study (4570) Determination of insulin icodec in human serum samples from "A Trial Investigating the Pharmacokinetic of insulin icodec in Subjects with Various Degrees of Hepatic Impairment" by an AlphaLisa method Study CA32983		
Assay passing rate	A total of 33 runs out of 34 (97.1%) were accepted according to the run acceptance criteria.	M 5.3.3.3, Trial 4570, Appendix 16.2.9, study CA32983, Table 1
Standard curve performance	• Cumulative bias range: -5.0% to 6.6 % • Cumulative precision: ≤5.6%	M 5.3.3.3, Trial 4570, Appendix 16.2.9, study CA32983, Table 2
QC performance	• Cumulative bias range: -2.5% to -0.8% • Cumulative precision: ≤10.0% • TE: not applicable	M 5.3.3.3, Trial 4570, Appendix 16.2.9, study CA32983, Table 8
Method reproducibility	Incurred sample re-analysis was performed in 68/502 (13.5%) of study samples, and 100% of the samples met the pre-specified criteria	M 5.3.3.3, Trial 4570, Appendix 16.2.9, study CA32983, Table 14
Study sample analysis/stability	Study samples, calibrators and QC's were all analysed within the validated long-term stability for 24 months at -20°C	M 5.3.3.3, Trial 4570, Appendix 16.2.9, study CA32983, Section 4.7.4 M 5.3.1.4, study 306660-CA25310-02, Table 8
Standard calibration curve performance during accuracy and precision runs	8 concentration levels and 3 anchor points 0[a,b], 250[b], 500, 1000, 3000, 10000, 20000, 40000, 64000, 80000 and 100000[b] (in pmol/L) [a]blank sample, [b]anchor point	
Method performance in study (4572) Determination of insulin icodec in human serum samples from "A Trial Investigating the Pharmacokinetic Properties of insulin icodec after Administration in Different Injections Regions in Subjects with Type 2 Diabetes" by an AlphaLisa method - Study CA32867-01		
Assay passing rate	A total of 91 runs out of 91 (100%) were accepted according to the run acceptance criteria.	M 5.3.1.1, Trial 4572, Appendix 16.2.9, study CA32867-01, Table 1

Standard curve performance	• Cumulative bias range: -6.0% to 7.3% • Cumulative precision: ≤3.9%	M 5.3.1.1, Trial 4572, Appendix 16.2.9, study CA32867-01, Table 2
QC performance	• Cumulative bias range: -1.7% to 1.7% • Cumulative precision: ≤6.9% • TE: not applicable	M 5.3.1.1, Trial 4572, Appendix 16.2.9, study CA32867-01, Table 4
Method reproducibility	Incurred sample re-analysis was performed in 142/1487 (9.5%) of study samples, and 99.3% of the samples met acceptance criteria	M 5.3.1.1, Trial 4572, Appendix 16.2.9, study CA32867-01, Table 8
Study sample analysis/stability	Study samples, calibrators and QC's were all analysed within the validated long-term stability for 24 months at -20°C	M 5.3.1.1, Trial 4572, Appendix 16.2.9, study CA32867-01, Section 4.7.4 M 5.3.1.4, study 306660-CA25310-02, Table 8
Standard calibration curve performance during accuracy and precision runs	8 concentration levels and 3 anchor points 0[a,b], 250b, 500, 1000, 3000, 10000, 20000, 40000, 64000, 80000 and 100000[b] (in pmol/L) [a]blank sample, [b]anchor point	
Method performance in study (4383) Determination of insulin 287 in human serum samples from "An investigational trial comparing the efficacy and safety of once weekly NNC0148-0287 C (insulin 287) versus once daily insulin glargine, both in combination with metformin, with or without DPP-4 inhibitors, in insulin naïve subjects with type 2 diabetes mellitus" by an AlphaLisa method - Study CA25157-01		
Assay passing rate	A total of 41 runs out of 46 (89.1%) were accepted according to the run acceptance criteria.	M 5.3.5.1, Trial 4383, Appendix 16.2.9, study CA25157-01, Table 1
Standard curve performance	• Cumulative bias range: -4.0% to 4.4% • Cumulative precision: ≤6.6%	M 5.3.5.1, Trial 4383, Appendix 16.2.9, study CA25157-01, Table 2
QC performance	• Cumulative bias range: 0.7% to 8.8%% • Cumulative precision: ≤12.9% • TE: not applicable	M 5.3.5.1, Trial 4383, Appendix 16.2.9, study CA25157-01, Table 6
Method reproducibility	Incurred sample re-analysis was performed in 94/854 (11.0%) of study samples, and 96.8% of the samples met acceptance criteria	M 5.3.5.1, Trial 4383, Appendix 16.2.9, study CA25157-01, Table 10
Study sample analysis/stability	Study samples, calibrators and QC's were all analysed within the validated long-term stability for 24 months at -20°C	M 5.3.5.1, Trial 4383, Appendix 16.2.9, study CA25157-01, Section 2.7.4 M 5.3.1.4, study 306660-CA25310-02, Table 8
Standard calibration curve performance during accuracy and precision runs	8 concentration levels and 3 anchor points 0[a,b], 250b, 500, 1000, 3000, 10000, 20000, 40000, 64000, 80000 and 100000[b] (in pmol/L) [a]blank sample, [b]anchor point	
Method performance in study (4478)		

Determination of insulin icodec in human serum samples from "A 26-week trial comparing the effect and safety of once weekly insulin icodec and once daily insulin degludec, both with or without non-insulin anti-diabetic drugs, in subjects with type 2 diabetes treated with basal insulin" by an AlphaLisa method - Study CA31565-01		
Assay passing rate	A total of 76 runs out of 77 (98.7%) were accepted according to the run acceptance criteria	M 5.3.5.1, Trial 4478, Bioanalytical Report, study CA31565-01, Table 1
Standard curve performance	• Cumulative bias range: -7.0% to 9.8% • Cumulative precision: ≤7.3%	M 5.3.5.1, Trial 4478, Bioanalytical Report, study CA31565-01, Table 2
QC performance	• Cumulative bias range: 0.0%- to -7.2% • Cumulative precision: ≤12.8% • TE: not applicable	M 5.3.5.1, Trial 4478, Bioanalytical Report, study CA31565-01, Table 6
Method reproducibility	Incurred sample re-analysis was performed in 136/1548 (8.79%) of study samples, and 91.9% of the samples met acceptance criteria	M 5.3.5.1, Trial 4478, Bioanalytical Report, study CA31565-01, Table 10
Study sample analysis/stability	Study samples, calibrators and QC's were all analysed within the validated long-term stability for 24 months at -20°C	M 5.3.5.1, Trial 4478, Bioanalytical Report, study CA31565-01, Section 4.7.4 M 5.3.1.4, study 306660-CA25310-02, Table 8
Standard calibration curve performance during accuracy and precision runs	8 concentration levels and 3 anchor points 0[a,b], 250[b], 500, 1000, 3000, 10000, 20000, 40000, 64000, 80000 and 100000[b] (in pmol/L) [a]blank sample, [b]anchor point	
Method performance in study (4479 ROW) Determination of insulin icodec in human serum samples from "A 26-week double blinded, multiregional, trial comparing the effect and safety of once weekly insulin icodec and once daily insulin degludec 100 units/mL, both in combination with non-insulin anti-diabetic drugs, in insulin naïve subjects with type 2 diabetes" by an AlphaLisa method - Study CA31566-01		
Assay passing rate	A total of 61 runs out of 62 (98.4%) were accepted according to the run acceptance criteria.	M 5.3.5.1, Trial 4479, Bioanalytical Report - Celerion, study CA31566-01, Table 1
Standard curve performance	• Cumulative bias range: -4.5% to 7.7% • Cumulative precision: ≤6.5%	M 5.3.5.1, Trial 4479, Bioanalytical Report - Celerion, study CA31566-01, Table 2
QC performance	• Cumulative bias range: -4.7% to -0.8% • Cumulative precision: ≤6.9% • TE: not applicable	M 5.3.5.1, Trial 4479, Bioanalytical Report - Celerion, study CA31566-01, Table 6
Method reproducibility	Incurred sample re-analysis was performed in 129/1432 (9.0%) of study samples, and 97.7% of the samples met acceptance criteria	M 5.3.5.1, Trial 4479, Bioanalytical Report - Celerion, study CA31566-01, Table 10

Study sample analysis/stability	Study samples, calibrators and QC's were all analysed within the validated long-term stability for 24 months at -20°C	M 5.3.5.1, Trial 4479, Bioanalytical Report - Celerion, study CA31566-01, Section 4.7.4 M 5.3.1.4, study 306660-CA25310-02, Table 8
Standard calibration curve performance during accuracy and precision runs	8 concentration levels and 3 anchor points 0[a,b], 250[b], 500, 1000, 3000, 10000, 20000, 40000, 64000, 80000 and 100000[b] (in pmol/L) [a]blank sample, [b]anchor point	
Method performance in study (4480) Determination of insulin icodec in human serum samples from "A 26-week trial comparing the effect and safety of once weekly insulin icodec and once daily insulin glargine 100 units/mL, both in combination with bolus insulin with or without non-insulin anti-diabetic drugs, in subjects with type 2 diabetes on a basal-bolus regimen." by an AlphaLisa method - Study CA31552-01		
Assay passing rate	A total of 76 runs out of 77 (98.7%) were accepted according to the run acceptance criteria.	M 5.3.5.1, Trial 4480, Bioanalytical Report, study CA31552-01, Table 1
Standard curve performance	• Cumulative bias range: -4.5% to 5.6% • Cumulative precision: ≤8.0%	M 5.3.5.1, Trial 4480, Bioanalytical Report, study CA31552-01, Table 2
QC performance	• Cumulative bias range: -6.0% to -1.7% • Cumulative precision: ≤11.2% • TE: not applicable	M 5.3.5.1, Trial 4480, Bioanalytical Report, study CA31552-01, Table 6
Method reproducibility	Incurred sample re-analysis was performed in 144/1662 (8.7%) of study samples, and 92.7% of the samples met the pre-specified criteria	M 5.3.5.1, Trial 4480, Bioanalytical Report, study CA31552-01, Table 10
Study sample analysis/stability	Study samples, calibrators and QC's were all analysed within the validated long-term stability for 24 months at -20°C	M 5.3.5.1, Trial 4480, Bioanalytical Report, study CA31552-01, Section 4.7.4 M 5.3.1.4, study 306660-CA25310-02, Table 8
Standard calibration curve performance during accuracy and precision runs	8 concentration levels and 3 anchor points 0[a,b], 250[b], 500, 1000, 3000, 10000, 20000, 40000, 64000, 80000 and 100000[b] (in pmol/L) [a]blank sample, [b]anchor point	
Method performance in study (4625 ROW) Determination of insulin icodec in human serum samples from "Efficacy and safety of once weekly insulin icodec compared to once daily insulin degludec 100 units/mL, both in combination with insulin aspart, in adults with type 1 diabetes. A 26-week, randomised, multicentre, open-label, active-controlled, parallel group, two armed, treat-to-target trial investigating the effect on glycaemic control and safety of treatment with once weekly insulin icodec compared to once daily insulin degludec, both in combination with insulin aspart in adults with type 1 diabetes, with a 26-week extension investigating long term safety." by an AlphaLisa method - Study CA33523-01		
Assay passing rate	A total of 64 runs out of 64 (100%) were accepted according to the run acceptance criteria.	M 5.3.5.1, Trial 4625, Bioanalytical Report, study CA33523-01, Table 1

Standard curve performance	• Cumulative bias range: -4.5 to 7.2% • Cumulative precision: ≤9.6%	M 5.3.5.1, Trial 4625, Bioanalytical Report, study CA33523-01, Table 2
QC performance	• Cumulative bias range: -6.0 to -0.8% • Cumulative precision: ≤7.0% • TE: not applicable	M 5.3.5.1, Trial 4625, Bioanalytical Report, study CA33523-01, Table 4
Method reproducibility	Incurred sample re-analysis was performed in 138/1545 (8.9%) of study samples, and 97.8% of the samples met the pre-specified criteria	M 5.3.5.1, Trial 4625, Bioanalytical Report, study CA33523-01, Table 7
Study sample analysis/stability	Study samples, calibrators and QC's were all analysed within the validated long-term stability for 24 months at -20°C	M 5.3.5.1, Trial 4625, Bioanalytical Report, study CA33523-01, Section 4.7.4 M 5.3.1.4, study 306660-CA25310-02, Table 8
Standard calibration curve performance during accuracy and precision runs	8 concentration levels and 3 anchor points 0[a,b], 250[b], 500, 1000, 3000, 10000, 20000, 40000, 64000, 80000 and 100000[b] (in pmol/L) [a]blank sample, [b]anchor point	

Bioanalytical method validation report name, amendments, and hyperlink	**Validation of an AlphaLisa method for the quantitative determination of insulin 0287 in human urine (M 5.3.1.4, study 306661-CA25312-01) and Partial Validation of an AlphaLisa method for the quantitative determination of insulin 0287 in human urine– Extension of Long Term Stability (M 5.3.1.4, study 306661-CA25312-02)**		
Method description	The LOCI method in serum was originally developed and validated at Novo Nordisk (M 5.3.1.4, study 212348 and study 216306), transferred to Celerion, Zürich, Switzerland and validated in human serum in study 306660-CA25310-01 and study 306660-CA25310-02. The LOCI method in urine was developed and validated by Celerion, Zürich, Switzerland (study 306661-CA25312-01) and extension of long-term stability was validated in study 306661-CA25312-02. VCA25312-01 is the primary validation report whereas long-term stability (at -20°C and -80°C) was assessed in partial-validation report VCA25312-02.		
Materials used for standard calibration curve and concentration	Human urine containing 0.1% Tween-20 0[a], 150, 300, 600, 1000, 2000, 5000, 8000, 10000 (all in pmol/L) [a]blank sample used as anchor point		
Validated assay range	150 – 10000 pmol/L		
Material used for Quality controls (QCs) and concentration	Human urine containing 0.1% Tween-20 LLOQ-QC:150, Low-QC:450, Medium-QC:2000, High-QC:7500, ULOQ-QC:10000, Dilution-QC:100000 (all in pmol/L)		
Minimum required dilution (MRDs)	10		
Source and lot of reagents	Biotinylated M-GIIone-2F4A80B65 Supplier: Novo Nordisk Batch: 15-May-2018/TiAd Conjugated acceptor beads with HUI-018 Supplier: Novo Nordisk Batch: HUI-018-270718/TiAd Streptavidin donor beads Supplier: PerkinElmer Batch: 2298681		
Regression model and weighting	5-parametric logistic (5-PL) regression with a $1/Y^2$ weighting factor		
Validation parameters	**Method validation summary**		**Source location**
Standard calibration curve performance during accuracy and precision runs	Number of calibrators from LLOQ to ULOQ	8	M 5.3.1.4, study 306661-CA25312-01, Table 2
	Cumulative accuracy (%bias) from LLOQ to ULOQ	-1.2% to 1.5%	M 5.3.1.4, study 306661-CA25312-01, Table 2 (for all accepted runs)
	Cumulative precision (%CV) from LLOQ to ULOQ	≤3.8%	
Performance of QCs during precision and accuracy runs	**Cumulative accuracy (%bias) in 5 QCs**	-1.4% to 9.4%	M 5.3.1.4, study 306661-CA25312-01, Table 8
	Inter-batch %CV	≤24.6%	

	Total error (TE)	≤34.0%	
Selectivity & matrix effect	Selectivity was performed in human urine at 0, 150 (LLOQ) and 7500 (high-QC) pmol/L in 10 individual samples Acceptance criteria were met at all levels		M 5.3.1.4, study 306661-CA25312-01, Table 9
Interference & specificity	Not applicable		Not applicable
Hemolysis effect	Not applicable		Not applicable
Lipemic effect	Not applicable		Not applicable
Dilution linearity & hook effect	Dilution linearity: Highest concentration tested at 100000 pmol/L in dilutions: undiluted, 1/2, 1/20, 1/40 and 1/80 The undiluted sample and 1/2 dilution were above ULOQ. All other dilutions met acceptance criteria: -18.5% to -8.8%bias Three duplicates at 1/40 dilution failed acceptance criteria: -20.6% to -20.5%bias Two samples at 1/80 dilution failed acceptance criteria: -22.1% to -21.4%bias Hook effect: No high dose hook effect was observed up to 100000 pmol/L.		M 5.3.1.4, study 306661-CA25312-01, Table 10
Bench-top/process stability	20h at RT		M 5.3.1.4, study 306661-CA25312-01, Table 11
Freeze-thaw stability	6 freeze/thaw cycles at -20°C and RT		
Long-term stability	at least 357 days at -20°C at least 357 days at -80°C		M 5.3.1.4, study 306661-CA25312-02, Table 5 (-20°C) and Table 6 (-80°C)
Parallelism	Not applicable		Not applicable
Carry over	Not applicable		Not applicable
Method performance in study (4226) Determination of insulin 287 in human urine samples from "Investigation of Pharmacokinetics and Safety Profile of a Single Dose NNC0148-0287 C (insulin 287) in Subjects with Various Degrees of Renal Impairment" by an AlphaLisa method - Study CA26323-02			
Assay passing rate	A total of 9 runs out of 9 (100%) were accepted according to the run acceptance criteria.		M 5.3.3.3, Trial 4226, Appendix 16.2.9, study CA26323-02, Table 1
Standard curve performance	• Cumulative bias range: -5.8% to 6.1% • Cumulative precision: ≤7.8%		M 5.3.3.3, Trial 4226, Appendix 16.2.9, study CA26323-02, Table 2
QC performance	• Cumulative bias range: -4.6% to 4.0% • Cumulative precision: ≤12.0% • TE: not applicable		M 5.3.3.3, Trial 4226, Appendix 16.2.9, study CA26323-02, Table 4

Method reproducibility	Incurred sample re-analysis was performed in 20/143 (14%) of study samples, and 95% of the samples met acceptance criteria	M 5.3.3.3, Trial 4226, Appendix 16.2.9, study CA26323-02, Table 6
Study sample analysis/stability	Study samples, calibrators, and QC's were all analysed within the validated long-term stability for 357 days at -20°C	M 5.3.3.3, Trial 4226, Appendix 16.2.9, study CA26323-02, Section 3.7.2 M 5.3.1.4, study 306661-CA25312-02, Table 5
Standard calibration curve performance during accuracy and precision runs	0[a], 150, 300, 600, 1000, 2000, 5000, 8000, 10000 (all in pmol/L) [a]blank sample used as anchor point	

Bioanalytical method validation report name, amendments, and hyperlink	**Validation of an Alphalisa for the quantification of insulin 287 in human serum (M 5.3.1.4, study 321497)**
Method description	The LOCI method was originally developed and validated at Novo Nordisk (M 5.3.1.4, study 212348 and study 216306). Prior to initiation of clinical trials in China, the assay was transferred to Wuxi Apptec Co., Shanghai, China, and validated in human serum, including cross-validation with sponsor supplied QC samples (study 321497). During this validation, the concentration of insulin icodec was used without taken purity into consideration although this was done in all other validations as well as reports from clinical trials. Two ranges are therefore listed below and validated in study 321497, Amendment 01: Analytical range C: 490.0 to 78740 pmol/L, all data from the validation recalculated, taking purity into consideration Analytical range D: 500 to 80000 pmol/L, analytical range to be used going forward (applies to reports from clinical trials). In addition, LTS and specificity is also validated in study 321497, Amendment 01. In study 321497, Amendment 02, extension of LTS and in-study selectivity (NN1436-4571) in T2D serum, parallelism is validated.
Materials used for standard calibration curve and concentration	Human serum 8 concentration levels and 2 anchor point **Analytical range C** 98090[a],78470, 62780, 39240, 19620, 9809, 2943, 981, 491 and 245[a] (all in pmol/L) [a]anchor point **Analytical range D** 100000[a], 80000, 64000, 40000, 20000, 10000, 3000, 1000, 500 and 250[a] (all in pmol/L) [a]anchor point
Validated assay range	**Analytical range C**: 491 to 78470 pmol/L **Analytical range D**: 500 to 80000 pmol/L
Material used for Quality controls (QCs) and concentration	Human serum **Analytical range C** LLOQ-QC:491, Low-QC:1471, Medium-QC:11770, High-QC:58850, ULOQ:78470, (all in pmol/L) **Analytical range D** LLOQ-QC:500, Low-QC:1500, Medium-QC:12000, High-QC:60000, ULOQ:80000, (all in pmol/L)
Minimum required dilution (MRDs)	50
Source and lot of reagents	Biotinylated M-GIIOne-2F4A80B65 Source: Novo Nordisk Batch: 280720/TiAd Conjugated acceptor beads with HUI-018 Source: Novo Nordisk Batch: 250520/TiAd and 071220/TiAd Streptavidin-coated Donor Beads Source: Perkin Elmer Batch: 2655617/ S000601/7

Regression model and weighting	5-parametric logistic (5-PL) regression with a $1/Y^2$ weighting factor		
Validation parameters	**Method validation summary**		**Source location**
Standard calibration curve performance during accuracy and precision runs	Number of calibrators from LLOQ to ULOQ	8	M 5.3.1.4, study 321497, Amendment 01, Table 10-1
	Cumulative accuracy (%bias) from LLOQ to ULOQ	range C: -1.3% to 5.1% range D: -1.5 to 3.2	M 5.3.1.4, study 321497, Amendment 01, Table 10-2 M 5.3.1.4, study 321497, Amendment 01, Table 10-13
	Cumulative precision (%CV) from LLOQ to ULOQ	range C: ≤5.1% range D: ≤2.4%	M 5.3.1.4, study 321497, Amendment 01, Table 10-2 M 5.3.1.4, study 321497, Amendment 01, Table 10-13
Performance of QCs during precision and accuracy runs	**Cumulative accuracy (%bias) in 5 QCs**	range C: -7.2% to -5.3% range D: -7.6% to -3.8%	M 5.3.1.4, study 321497, Amendment 01, Table 10-4 M 5.3.1.4, study 321497, Amendment 01, Table 10-15
	Inter-batch %CV	range C: ≤13.0% range D: ≤9.1%	M 5.3.1.4, study 321497, Amendment 01, Table 10-4 M 5.3.1.4, study 321497, Amendment 01, Table 10-15
	Total error (TE)	range C: ≤16.9% range D: ≤15.1%	M 5.3.1.4, study 321497, Amendment 01, Table 10-4 M 5.3.1.4, study 321497, Amendment 01, Table 10-15
Selectivity & matrix effect	Selectivity was performed in human serum at 490.5 pmol/LM (LLOQ) and 58850 (high-QC) pmol/L in three populations with 10 individual samples in each population Normal human serum 10 out of 10 passed: -2.2% to 4.9%bias Normal Chinese serum 9 out of 10 passed: -17.7% to 8.4%bias 1 out of 10 failed at high-QC: -23.2%bias Selectivity in T2D serum was investigated in-study (trial 4571) and performed at 0, 500 (LLOQ) and 60000 (high-QC) pmol/L with 10 individual pre-dose samples.		M 5.3.1.4, study 321497, Amendment 01, Section 4.4 M 5.3.1.4, study 321497, Amendment 01, Table 10-6

	T2D serum 10 out of 10 passed: -21.6% to 15.5%bias	M 5.3.1.4, study 321497, Amendment 02, Table 10-17
Interference & specificity	Specificity of the assay was tested with the following compounds: human insulin, insulin aspart, insulin degludec, insulin detemir, insulin glargine and semaglutide at LLOQ and ULOQ levels of insulin icodec Acceptance criteria were met for each concentration of each analogue	M 5.3.1.4, study 321497, Amendment 01, Table 10-12
Hemolysis effect	3 lots tested with 10% hemolysis degree, all meet the acceptance criteria: -7.9% to -2.0%bias	M 5.3.1.4, study 321497, Amendment 01, Table 10-6
Lipemic effect	3 lots tested, all meet the acceptance criteria: -3.6% to 6.9%bias	
Dilution linearity & hook effect	Dilution linearity: Highest concentration tested at 1962000 pmol/L in dilutions:1/25, 1/250, 1/2500 and 1/20000 The dilution 1/20000 was below LLOQ. All other dilutions met over-all acceptance criteria. 2 duplicates did not meet acceptance criteria: -22.0% and -21.0% Dilution linearity up to: 1/2500 dilution Hook effect: No hook effect observed up to 1962000 pmol/L	M 5.3.1.4, study 321497, Amendment 01, Table 10-7 M 5.3.1.4, study 321497, Amendment 01, Table 10-8
Bench-top/process stability	Human serum 24h at RT 24h at 2°C to 8°C	M 5.3.1.4, study 321497, Amendment 01, Table 10-10
Freeze-thaw stability	5 freeze-thaw cycles at -70°C	
Long-term stability	505 days at -20°C 505 days at -70°C	M 5.3.1.4, study 321497, Amendment 02, Table 10-18
Parallelism	Parallelism investigated in-study, trial 4571: 3 samples near high-QC, %CV ≤4.9% across dilutions and across samples	M 5.3.1.4, study 321497, Amendment 02, Table 10-19
Carry over	Not applicable	Not applicable
Method performance in study 4479 China NN1436-4479: determination of the Concentration of insulin icodec in hunman Serum by Alphalisa, in support of the clinical Study referenced NN1436-4479 - Study 407279-214197-PSA-HGR		
Assay passing rate	A total of 15 runs out of 17 (88.2%) were accepted according to the run acceptance criteria.	M 5.3.5.1, Trial 4479, Bioanalytical Report - Wuxi, study 407279-214197-PSA-HGR, Appendix A
Standard curve performance	• Cumulative bias range: -1.1% to 4.0% • Cumulative precision: ≤8.7%	M 5.3.5.1, Trial 4479, Bioanalytical Report - Wuxi, study 407279-214197-PSA-HGR, Appendix C

QC performance	• Cumulative bias range: -1.3% to 1.5% • Cumulative precision: ≤11.2% • TE: not available	M 5.3.5.1, Trial 4479, Bioanalytical Report - Wuxi, study 407279-214197-PSA-HGR, Appendix E
Method reproducibility	Incurred sample re-analysis was performed in 29/270 (10.7%) of study samples, and 96.6% of the samples met acceptance criteria	M 5.3.5.1, Trial 4479, Bioanalytical Report - Wuxi, study 407279-214197-PSA-HGR, Appendix J
Study sample analysis/stability	Study samples, calibrators and QC's were all analysed within the validated long-term stability for 24 months at -20°C	M 5.3.5.1, Trial 4479, Bioanalytical Report - Wuxi, study 407279-214197-PSA-HGR, Section Brief Summary - Bioanalysis M 5.3.1.4, study 321497, Amendment 02, Table 10-18
Standard calibration curve performance during accuracy and precision runs	8 concentration levels and 2 anchor point 100000[a], 80000, 64000, 40000, 20000, 10000, 3000, 1000, 500 and 250[a] (all in pmol/L) [a]anchor point	
Method performance in study 4571 NN1436-4571: determination of the Concentration of insulin icodec in human Serum by Alphalisa, in support of clinical study Referenced NN1436-4571 - Study 407279-214227-PSA-HGR		
Assay passing rate	A total of 54 runs out of 55 (98.2%) were accepted according to the run acceptance criteria.	M 5.3.3.3, Trial 4571, Appendix 16.2.9, study 407279-214227-PSA-HGR, Appendix A
Standard curve performance	• Cumulative bias range: -1.2% to 3.9% • Cumulative precision: ≤6.6%	M 5.3.3.3, Trial 4571, Appendix 16.2.9, study 407279-214227-PSA-HGR, Appendix C
QC performance	• Cumulative bias range: -4.3% to 2.2% • Cumulative precision: ≤16.8% • TE: not applicable	M 5.3.3.3, Trial 4571, Appendix 16.2.9, study 407279-214227-PSA-HGR, Appendix D
Method reproducibility	Incurred sample re-analysis was performed in 95/961 (9.9%) of study samples, and 96.8% of the samples met acceptance criteria	M 5.3.3.3, Trial 4571, Appendix 16.2.9, study 407279-214227-PSA-HGR, Appendix G
Study sample analysis/stability	Study samples, calibrators and QC's were all analysed within the validated long-term stability for 24 months at -20°C	M 5.3.3.3, Trial 4571, Appendix 16.2.9, study 407279-214227-PSA-HGR, Section Brief Summary - Bioanalysis M 5.3.1.4, study 321497, Amendment 02, Table 10-18
Standard calibration curve performance during accuracy and precision runs	8 concentration levels and 2 anchor point 100000[a], 80000, 64000, 40000, 20000, 10000, 3000, 1000, 500 and 250[a] (all in pmol/L) [a]anchor point	

Table 4.1.4 Summary of Method Modifications and Cross-validation Results

Bioanalytical method validation report name and hyperlink	**Partial Validation of an AlphaLisa method for the quantitative determination of insulin 0287 in human serum – Extension of Long Term Stability, method automation, cross-reactivity with insulin degludec and cross-validation with sponsor supplied QC samples (M 5.3.1.4, study 306660-CA25310-02)**		
Changes in method	The LOCI method was originally developed and validated at Novo Nordisk (M 5.3.1.4, study 212348 and study 216306). Prior to initiation of clinical trials, the assay was transferred to Celerion and validated, including cross-validation with sponsor supplied QC's (study 306660-CA25310-02). Cross-validation: The assay was transferred from Novo Nordisk to Celerion, where calibrators and QC samples were delivered from sponsor and bridged at Celerion within acceptance criteria in a formal cross-validation.		
New validated assay range if any	500 - 80000 pmol/L		
Validation parameters	**Cross-validation performance**		**Source location**
Standard calibration curve performance during accuracy and precision runs	Cumulative accuracy (%bias) in standard calibrators from LLOQ to ULOQ	Range C: -2.7% to 4.3%	M 5.3.1.4, study 306660-CA25310-02, Table 2
	Cumulative precision (%CV) from LLOQ to ULOQ	range C: ≤6.0%	
Performance of QCs during accuracy and precision runs	**Cumulative accuracy (%Bias) in 5 QCs**	not applicable	not applicable
	Inter-batch %CV	not applicable	not applicable
	Percent TE	not applicable	not applicable
Cross-validation	QC samples at 3 levels were prepared in 6 replicates and accepted by sponsor prior to analysis at Celerion (M 5.3.1.4, study 306660-CA25310-02, Table 13) Accuracy (%bias) Low-QC: 1.1% to 7.3% Medium-QC: 5.0% to 9.7% High-QC: -2.2% to 10.5% Precision (%CV) Low-QC: overall 2.0% Medium-QC: overall 1.8% High-QC: overall 4.5%		
List other parameters	Not applicable		

Bioanalytical method validation report name and hyperlink	**NNC0148-287: Validation of an AlphaLisa for the quantification of insulin 287 in human serum (M 5.3.1.4, study 321497)**		
Changes in method	The LOCI method was originally developed and validated at Novo Nordisk (M 5.3.1.4, study 212348 and study 216306). Prior to initiation of clinical trials in China, the assay was transferred to Wuxi Apptec Co., Shanghai, China and validated, including cross-validation with sponsor supplied QC's (study 321497). Cross-validation: The assay was transferred from Novo Nordisk to Wuxi Apptec, where calibrators and QC samples were delivered from sponsor and bridged at Wuxi Apptec within acceptance criteria in a formal cross-validation.		
New validated assay range if any	500 - 80000 pmol/L		
Validation parameters	**Cross-validation performance**		**Source location**
Standard calibration curve performance during accuracy and precision runs	Cumulative accuracy (%bias) in standard calibrators from LLOQ to ULOQ	Range C: -1.3% to 5.1%	M 5.3.1.4, study 321497, Amendment 01, Table 10-2
	Cumulative precision (%CV) from LLOQ to ULOQ	range C: ≤5.1%	
Performance of QCs during accuracy and precision runs	**Cumulative accuracy (%Bias) in 5 QCs**	range C: -7.2% to -5.3%	M 5.3.1.4, study 321497, Amendment 01, Table 10-4
	Inter-batch %CV	range C: ≤13.0%	
	Percent TE	range C: ≤16.9 %	
Cross-validation	QC samples at 3 levels were prepared in 5 replicates and accepted by sponsor prior to analysis at Wuxi. The 5 sets of each QC sample were analysed in duplicates at Wuxi (M 5.3.1.4, study 321497, Amendment 01, Table 10-9) Accuracy (%bias) Low-QC: -16.8% to -4.0% Medium-QC: -13.4% to -1.9% High-QC: -18.5% to -8.4% Precision (%CV) Low-QC: ≤1.6% Medium-QC: ≤4.3% High-QC: ≤19.1%		
List other parameters	Not applicable		