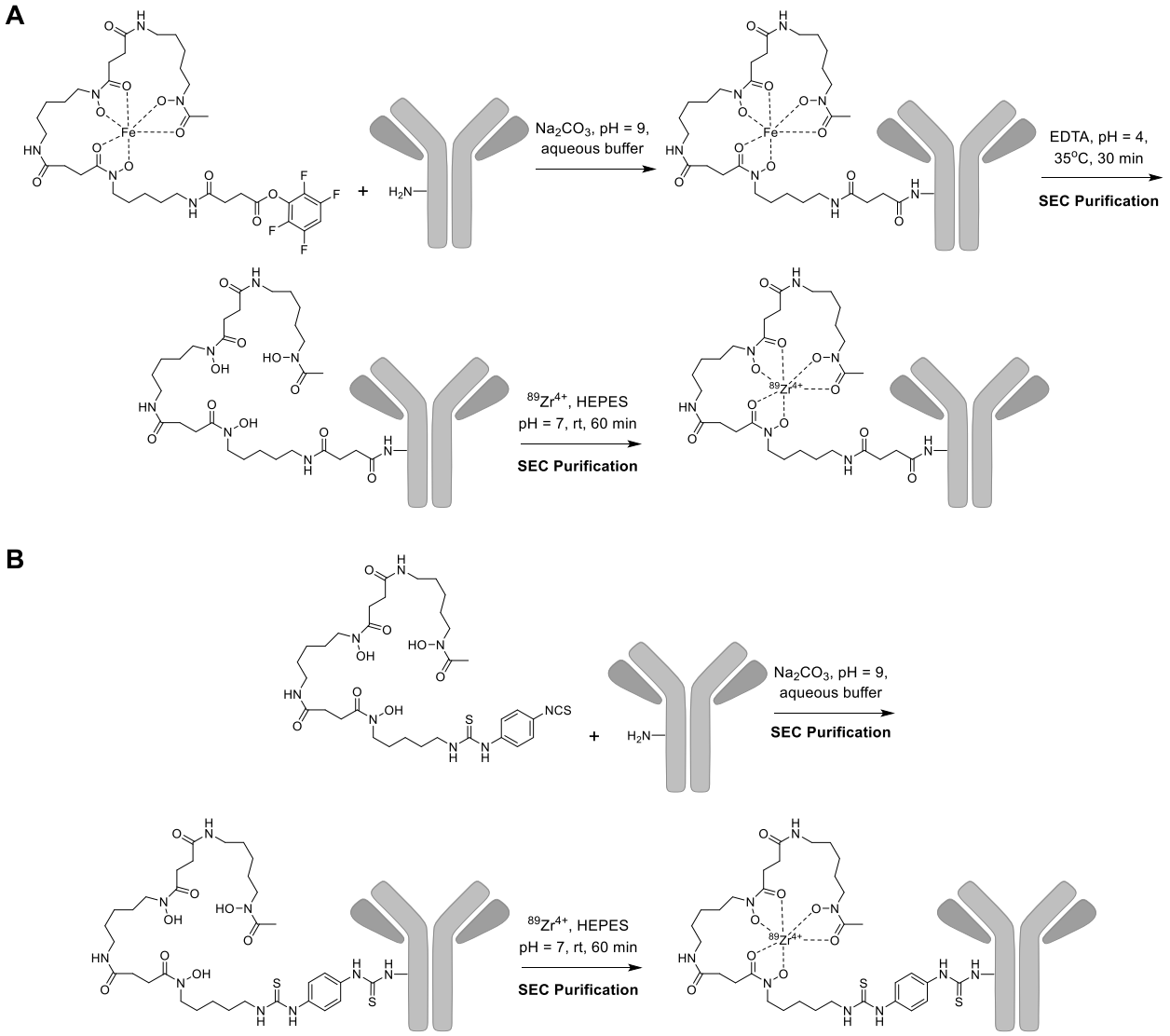
**Reaction scheme of $^{89}$Zr-mAb labeling**




**Scheme 1:** Methods for $^{89}$Zr-mAb labeling for clinical use to date: **(A)** Modification of a mAb with TFP-*N*-suc-Df-Fe, followed by the removal of Fe and the $^{89}$Zr-labeling of the DFO-mAb in the final step. **(B)** Modification of a mAb with Df-Bz-NCS, followed by the $^{89}$Zr-labeling of the DFO-mAb in the final step.

**General**: All reagents and solvents were purchased from Sigma Aldrich. Disposable tubing with luer locks were obtained from Vygon (Valkenswaard. The Netherlands). Disposable syringes were obtained from BD Plastipak (Franklin Lakes. USA). Cetuximab (5 mg/mL) directed against the epidermal growth factor receptor (EGFR) and rituximab (10 mg/mL) directed against CD20. were obtained from the VU University Medical Center pharmacy. In this study. TFP-*N*-suc-Df-Fe has been prepared in-house and is stored in acetonitrile at -80˚C.[1] The chemical modification to obtain DFO-*N*-suc-cetuximab and DFO-*N*-suc-Rituximab for radiolabeling has been performed according to literature procedures.[11. 19] Size exclusion (SEC)-HPLC was performed to determine protein integrity and radiochemical purity on a Jasco HPLC system (Easton. USA) equipped with a superdex 200 10/30 GL size exclusion column (GE Healthcare Life Sciences. Eindhoven. The Netherlands) including a guard column. using a mixture of 0.05 M sodium phosphate. 0.15 M sodium chloride (pH 6.8). and 0.01 M $NaN_3$ as the eluent at a flow rate of 0.5 mL/min. The mAb is eluted at 25 minutes. whereas free $^{89}$Zr is eluted at 45 minutes using this HPLC setup. The radioactivity of the eluate was monitored using an in-line NaI(Tl) radiodetector (Raytest Sockett). iTLC analysis was performed to determine the radiolabeling efficiency and to determine the product radiochemical purity after PD-10 purification (GE Healthcare Life Sciences. Eindhoven. The Netherlands). in addition to SEC-HPLC. TLC was performed with iTLC strips (TEC-Control. Chromatography Strips. Model 150-771) and run with MeCN/20 mM citric acid pH 4.8 – 5.2 (1:9. *v/v*). The immunoreactivity was determined using A431 (EGFR) or SU-DHL-4 (CD20) cells. essentially as described by Lindmo *et al.*[2] Endotoxin content was tested using a Endosafe-PTS100 reader from Charles Rivers (Wilmington. USA). $^{89}$Zr with a molar activity of ≥ 0.15 GBq/nmol in 1 M oxalic acid was obtained from Perkin Elmer (Boston. USA). The new radiopharmaceutical chemistry nomenclature guidelines were followed.[3]

**Detailed System Setup**: At the indicated valve positions on the cassettes the following was mounted (Figure 1):

1. A Lectro-cath polyethylene tubing 50 cm (Vygon) is connecting cassette 1 to valve 6 of cassette 2.
2. A 3 mL syringe luer lock tip (BD Plastipak. 300185) containing a solution of DFO-mAb: DFO-*N*-suc-cetuximab or DFO-*N*-suc-rituximab (0.71 mL. 2 – 5 mg/mL) and 0.5 M HEPES pH = 7 (0.5 mL) to valve 2.
3. A 3 mL syringe luer lock tip (BD Plastipak. 300185) containing a solution of 2 M $Na_2CO_3$ (0.09 mL) and 0.5 M HEPES pH = 7 (0.5 mL) to valve 3.
4. A 5 mL syringe luer lock tip (BD Plastipak. 302187) containing an eluent solution of 5 mg/mL gentisic acid pH = 5.0 (3.0 mL for cetuximab and 2.5 mL for rituximab) to valve 4.
6. In horizontal position a V-green 10 cm polyethylene tubing (Vygon) is connecting a needle ventilated glass vial containing $^{89}$Zr in 1M oxalic acid (0.2 mL) to valve 6.
7. A Lectro-cath polyethylene tubing 15 cm (Vygon) is connecting a 20 mL syringe luer lock tip (Braun) to valve 7.

8. A Lectro-cath polyethylene tubing 15 cm (Vygon) is connecting valve 8 to a PD-10 desalting column on valve 10.
9. A V-green 10 cm polyethylene tubing (Vygon) equipped with a GS 0.22 um filter unit (Millex) is connected to valve 9 for ventilation.
10. In vertical position a PD-10 desalting column is mounted. preconditioned by flushing with 5 mg/mL gentisic acid pH = 5.0 in saline (4 x 5 mL). while in horizontal position a Lectro-cath polyethylene tubing 15 cm (Vygon) is placed connecting a needle ventilated sterile glass vial capped with rubber septum (20 mL) for product collection to valve 10.

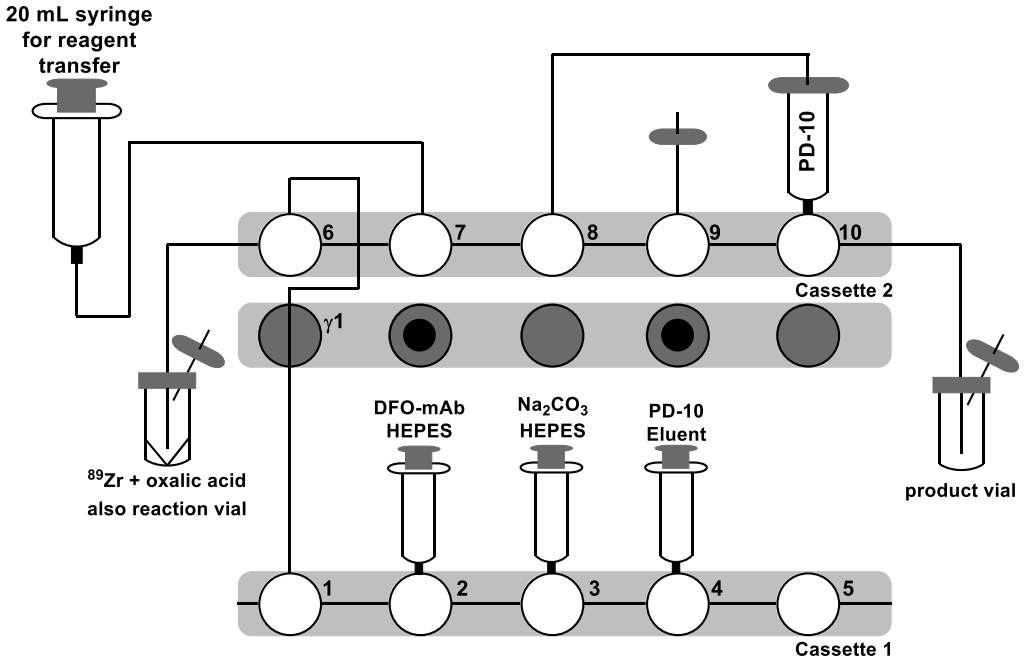



**Figure 1:** Schematic representation of the complete and optimized automated $^{89}$Zr-labeling of DFO-mAb on a Scintomics GRP 2V module with the positions of reagent vials. $^{89}$Zr-containing reaction vial. transfer lines. PD-10 desalting column and product vial that will contain the purified $^{89}$Zr-labeled mAb indicated.

**Full Quality control**: To determine the radiolabeling yield the start amount of radioactivity. which is present in the vial that contains $^{89}$Zr in 1M oxalic acid. and the amount of radioactivity in the product vial after PD-10 purification were measured in a dose calibrator. To calculate the percentage radiolabeling yield. the amount of radioactivity in the product vial was divided by the start amount of radioactivity and multiplied by 100%.[3]

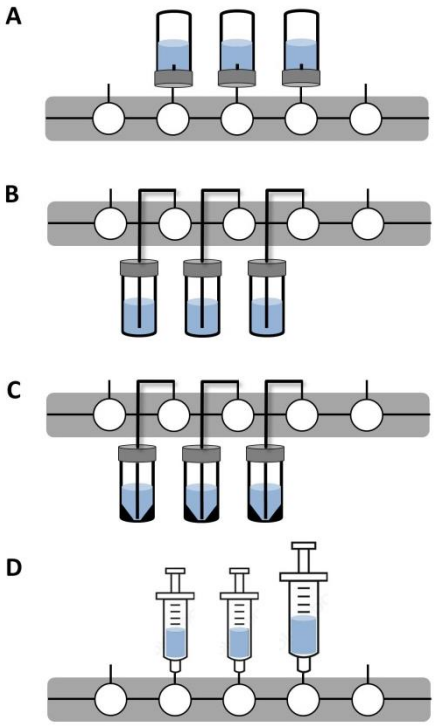
For radiochemical purity analysis by iTLC. 2 µL of the product fraction was spotted on an iTLC strip and developed with MeCN/20 mM citric acid (1:9. *v/v*). [$^{89}$Zr]Zr-DFO-*N*-suc-cetuximab and [$^{89}$Zr]Zr-DFO-*N*-suc-rituximab had a Rf = 0. and free $^{89}$Zr and [$^{89}$Zr]Zr-*N*-suc-DFO a Rf = 1. When the eluent front reached the top of the iTLC strip. the strip was taken from the eluent. and cut on the indicated line. The bottom and top of the strip were counted

independently in a γ-counter (LKB Wallac 1282 CompuGamma; Pharmacia) on the 511 and 909-keV γ-energy of $^{89}$Zr. The radiochemical purity was calculated by dividing the product counts by the total number of counts from the iTLC strip. multiplied by 100%.

For radiochemical purity analysis by SEC-HPLC. 20 µL of the product fraction was injected onto a size-exclusion HPLC column. UV absorption was detected at a 280 nm wavelength and the radioactivity signal by radio detection (Figure 1). The $^{89}$Zr-labeled mAbs elute after approximately 25 minutes from the HPLC column. whereas unconjugated $^{89}$Zr and [$^{89}$Zr]Zr-*N*-suc-DFO elutes after approximately 45 minutes. All peaks were integrated to determine the radiochemical purity according to SEC-HPLC, as well as the protein integrity.

The immunoreactivity was determined essentially as described by Lindmo et al. using EGFR-expressing fixed A431 cells for [$^{89}$Zr]Zr-DFO-*N*-suc-cetuximab and CD20-expressing fixed SU-DHL4 cells for [$^{89}$Zr]Zr-DFO-*N*-suc-rituximab.[2]

**Tested vial configurations for automated $^{89}$Zr-mAb labeling**




Schematic representation of tested vial configurations for optimal reagent aspiration and transfer to the reaction vial with **(A)** Up-side-down vials with purged septum and excess of reagents; **(B)** flat-bottom vials containing excess of reagents; **(C)** Successful setup with V-shaped vials with exact reagent volumes and **(D)** Successful setup with syringes with exact reagent volumes.

**Labeling data of 20 MBq reactions for [$^{89}$Zr]Zr-DFO-N-suc-cetuximab and [$^{89}$Zr]DFO-N-suc-rituximab**

**Data of produced [$^{89}$Zr]Zr-DFO-N-suc-cetuximab:**

PD-10 Elution: 2 mL reaction mixture
1 mL flow through
2.5 mL product elution

All three are combined in 1 product fraction.

Average yields base on iTLC of 3 productions of [$^{89}$Zr]Zr-DFO-N-suc-cetuximab: 90.3 ± 10.5 %
Average isolated yields of the 3 productions of [$^{89}$Zr]Zr-DFO-N-suc-cetuximab: 73.8 ± 5.3 %

Table 1: overview the automated [$^{89}$Zr]Zr-DFO-N-suc-cetuximab labeling using a Scintomics module

| **Labeling number** | **1** | **2** | **3** |
|---|---|---|---|
| | | | |
| Start activity (MBq) | 21.45 | 20.74 | 20.4 |
| Isolated product (MBq) | 14.55 | 16.11 | 15.47 |
| Labeling yield (non decay corrected )(%) | 67.8 | 77.7 | 75.8 |
| | | | |
| Radiochemical yield by iTLC of the crude reaction mixture (%) | 78.2 | 96.1 | 96.5 |
| | | | |
| Radiochemical purity by iTLC (%) | 98.9 | 98.9 | 98.5 |
| Radiochemical purity by SEC-HPLC (%) | 99.3 | 99.7 | 99.6 |

**Data of produced [$^{89}$Zr]Zr-DFO-N-suc-rituximab:**

PD-10 Elution: 2 mL reaction mixture
1mL flow through
2 mL product elution

All three are combined in 1 product fraction.

Average yields based on iTLC of 3 productions of [$^{89}$Zr]Zr-DFO-N-suc-cetuximab: 96.0 ± 0.5 %
Average isolated yields of 3 productions of [$^{89}$Zr]Zr-DFO-N-suc-rituximab: 62.8 ± 5.1 %

Table 2: overview the automated [$^{89}$Zr]Zr-DFO-N-suc-rituximab labeling using a Scintomics Module

| **Labeling number** | **1** | **2** | **3** |
|---|---|---|---|
| | | | |
| Start activity (MBq) | 17.45 | 17.15 | 16.05 |
| Isolated product (MBq) | 10.85 | 11.69 | 9.30 |
| Labeling yield (non decay corrected )(%) | 62.3 | 68.2 | 58.0 |
| | | | |
| Radiochemical yield by iTLC of the crude reaction mixture (%) | 96.1 | 96.5 | 95.5 |
| | | | |
| Radiochemical purity by iTLC (%) | 99.3 | 99.0 | 99.2 |
| Radiochemical purity by SEC-HPLC (%) | 100.0 | 100.0 | 100.0 |

**Labeling data of 60 MBq reactions for [$^{89}$Zr]Zr-DFO-N-suc-cetuximab and [$^{89}$Zr]DFO-N-suc-rituximab**

**Data of produced [$^{89}$Zr]Zr-DFO-N-suc-cetuximab:**

Table 3: Production and Quality Control data

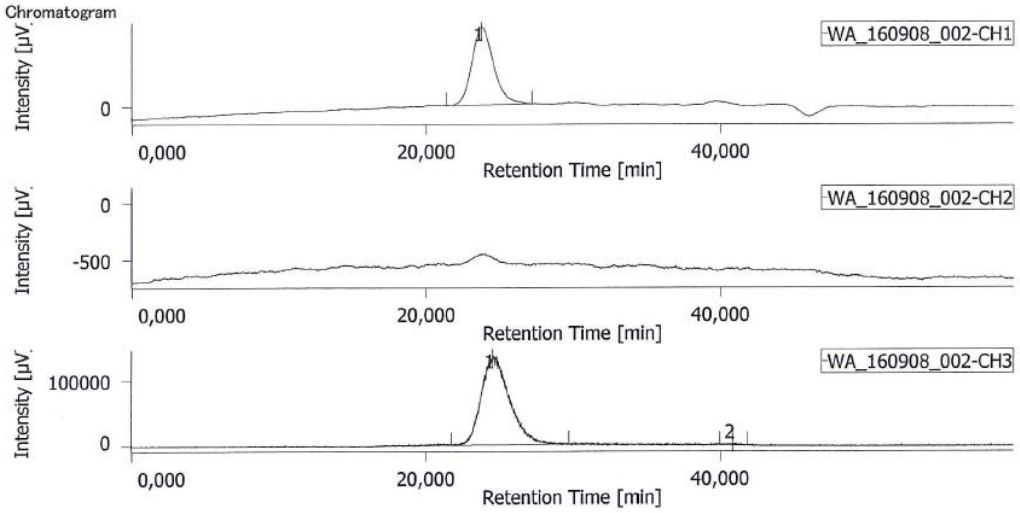
| **Labeling number** | **1** | **2** | **3** |
|---|---|---|---|
| **Reaction details** | | | |
| Start activity (MBq) | 63.03 | 65.88 | 61.71 |
| Radiochemical yield by iTLC of the crude reaction mixture. before PD-10 purification (%) | 93.0 | 92.3 | 90.7 |
| Isolated product (MBq) | 46.67 | 50.59 | 44.97 |
| Labeling yield (non decay corrected) (%) | 74.0 | 76.8 | 72.9 |
| | | | |
| **Quality Control purified product** | | | |
| Radiochemical purity by iTLC (%) | 98.2 | 98.7 | 98.0 |
| Radiochemical purity by SEC-HPLC (%) | 99.3 | 99.4 | 99.8 |
| Protein integrity by SEC-HPLC at 280 nm (%) | 100.0 | 100.0 | 100.0 |
| Immunoreactivity by Lindmo assay on A431 cells (%) | 96.6 | 95.1 | 95.5 |
| | | | |
| **Endotoxin tests** | | | |
| Sample value bacterial endotoxin content assay (< 2.5 EU/mL) | 0.278 | 0.200 | 0.200 |
| Sample rxn time CV bacterial endotoxin content assay (< 25%) | 19.1 | 0.0 | 0.0 |
| Spike rxn time CV bacterial endotoxin content assay (< 25%) | 2.2 | 1.3 | 3.8 |
| Spike recovery bacterial endotoxin content assay (50 -200%) | 90 | 108 | 96 |

**Data of produced [$^{89}$Zr]Zr-DFO-N-suc-rituximab:**

Table 4: Production and Quality Control data

| **Labeling number** | **1** | **2** | **3** |
|---|---|---|---|
| **Reaction details** | | | |
| Start activity (MBq) | 57.14 | 66.54 | 65.34 |
| Radiochemical yield by iTLC of the crude reaction mixture. before PD-10 purification (%) | N.D. | 95.3 | 95.4 |
| Isolated product (MBq) | 33.83 | 43.28 | 41.56 |
| Labeling yield (non decay corrected) (%) | 59.2 | 65.0 | 63.6 |
| | | | |
| **Quality Control purified product** | | | |
| Radiochemical purity by iTLC (%) | 99.1 | 99.2 | 99.1 |
| Radiochemical purity by SEC-HPLC (%) | 100.0 | 100.0 | 100.0 |
| Protein integrity by SEC-HPLC at 280 nm (%) | 100.0 | 100.0 | 100.0 |
| Immunoreactivity by Lindmo assay on A431 cells (%) | 86.8 | 87.2 | 89.5 |
| | | | |
| **Endotoxin tests** | | | |
| Sample value bacterial endotoxin content assay (< 2.5 EU/mL) | 0.503 | 0.200 | 0.200 |
| Sample rxn time CV bacterial endotoxin content assay (< 25%) | 0.0 | 0.0 | 0.0 |
| Spike rxn time CV bacterial endotoxin content assay (< 25%) | 13.3 | 5.1 | 3.3 |
| Spike recovery bacterial endotoxin content assay (50 -200%) | 99 | 122 | 133 |

**SEC-HPLC of [$^{89}$Zr]Zr-DFO-N-suc-cetuximab produced on a Scintomics automate**



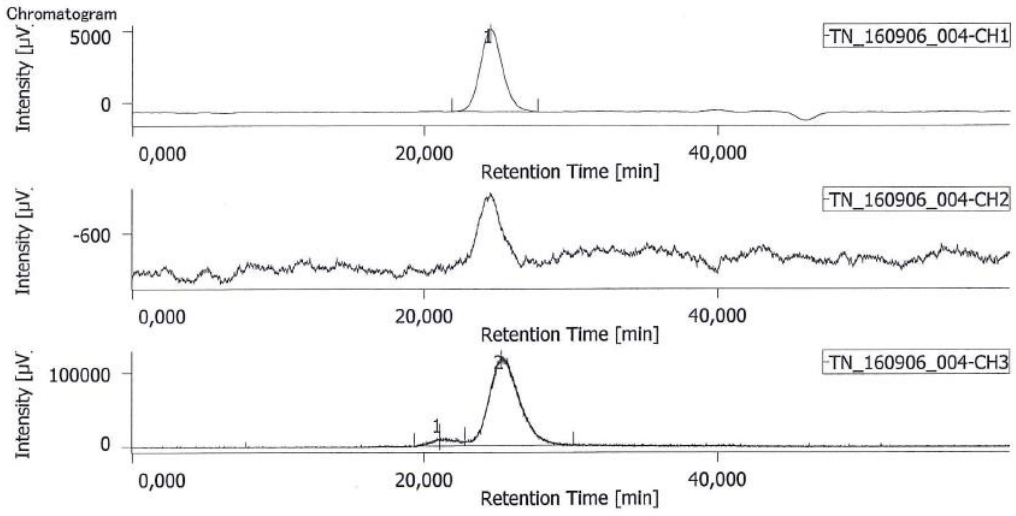

Peak Information

| # | Peak Name | CH | tR [min] | Area [μV·sec] | Height [μV] | Area% | Peak Width (half height) | Resolution |
|---|---|---|---|---|---|---|---|---|
| 1 | mAb | 1 | 23,750 | 423762 | 4134 | 100,000 | 1,542 | N/A |

Peak Information WA_160908_002 CH-2 – No data.

| # | Peak Name | CH | tR [min] | Area [μV·sec] | Height [μV] | Area% | Peak Width (half height) | Resolution |
|---|---|---|---|---|---|---|---|---|
| 1 | Unknown | 3 | 24,492 | 18009088 | 138186 | 99,440 | 1,991 | 5,956 |
| 2 | Unknown | 3 | 40,783 | 101473 | 3639 | 0,560 | 1,237 | N/A |

**SEC-HPLC of [$^{89}$Zr]Zr-DFO-N-suc-rituximab produced on a Scintomics automate**



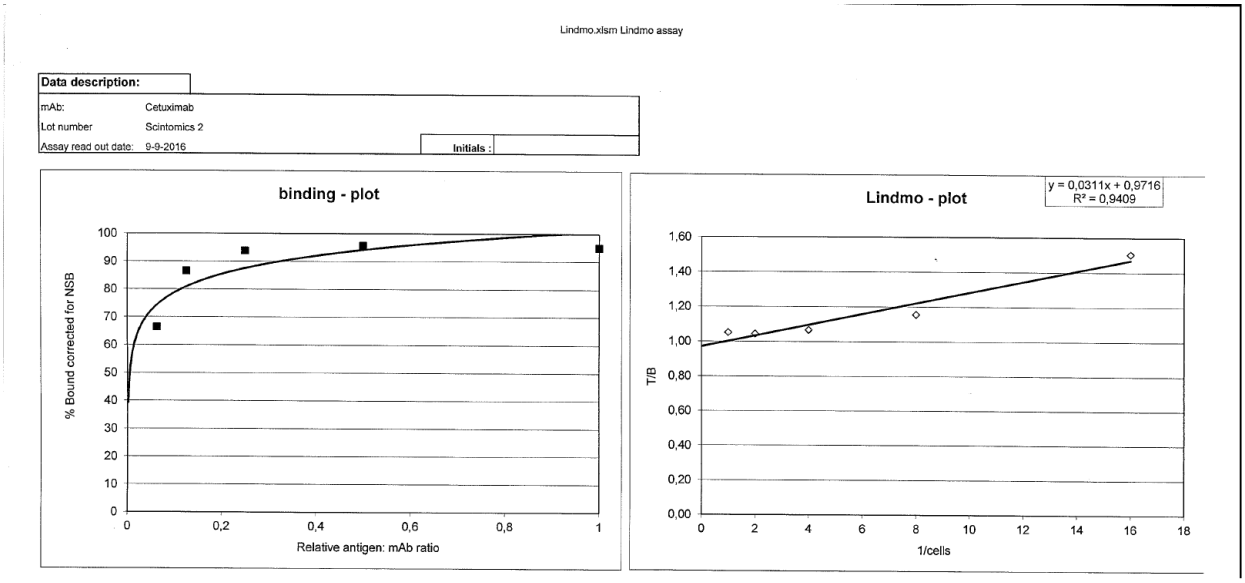

Peak Information

| # | Peak Name | CH | tR [min] | Area [μV·sec] | Height [μV] | Area% | Peak Width (half height) | Resolution |
|---|---|---|---|---|---|---|---|---|
| 1 | Unknown | 1 | 24,500 | 603753 | 5734 | 100,000 | 1,607 | N/A |

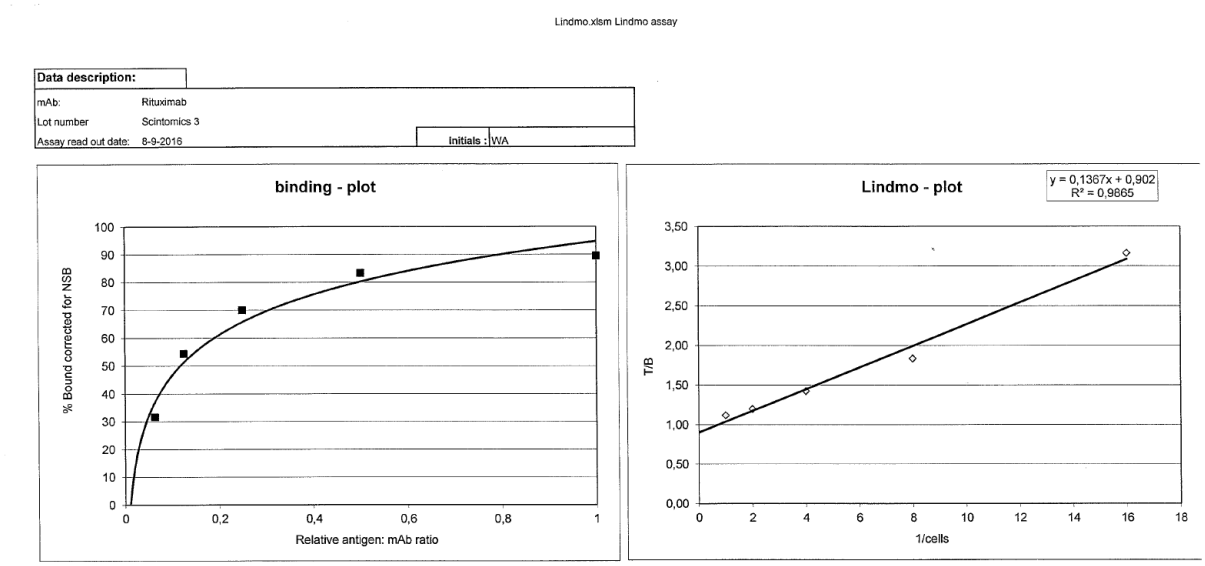
Peak Information TN_160906_004 CH-2 – No data.

| # | Peak Name | CH | tR [min] | Area [μV·sec] | Height [μV] | Area% | Peak Width (half height) | Resolution |
|---|---|---|---|---|---|---|---|---|
| 1 | Unknown | 3 | 21,058 | 1140908 | 11987 | 6,336 | 2 625 | 1,038 |
| 2 | Unknown | 3 | 25,250 | 16865046 | 122011 | 93,664 | 2 139 | N/A |

**Lindmo binding curve of [$^{89}$Zr]Zr-DFO-N-suc-cetuximab produced on a Scintomics automate**




**Lindmo binding curve of [$^{89}$Zr]Zr-DFO-N-suc-rituximab produced on a Scintomics automate**



**Program for automated labeling**

| Time [min] | Function | Parameter |
|---|---|---|
| 0.00 | Start Chromatogram | Ch. 4.5.6.7.9.10 ( 500 ms SliceWidth ) |
| 0.02 | Valve Position | Valve 7 = Position 4 |
| 0.02 | Valve Position | Valve 6 = Position 2 |
| 0.02 | Valve Position | Valve 3 = Position 4 |
| 0.02 | Valve Position | Valve 2 = Position 3 |
| 0.02 | Valve Position | Valve 1 = Position 2 |
| 0.05 | Dispenser (Dispenser 1) | Dispenser 1 Aspirates 3000 µl from 1 (3000 µl/min) |
| 1.06 | Wait for Input | Signal Dispenser 1 ready |
| 1.08 | Valve Position | Valve 6 = Position 3 |
| 1.10 | Dispenser (Dispenser 1) | Dispenser 1 Dispenses 3000 µl to 1 (3000 µl/min) |
| 2.11 | Wait for Input | Signal Dispenser 1 ready |
| 2.13 | Dispenser (Dispenser 1) | Dispenser 1 Aspirates 3000 µl from 1 (4000 µl/min) |
| 2.89 | Wait for Input | Signal Dispenser 1 ready |
| 2.91 | Dispenser (Dispenser 1) | Dispenser 1 Dispenses 3000 µl to 1 (4000 µl/min) |
| 3.67 | Wait for Input | Signal Dispenser 1 ready |
| 3.69 | Valve Position | Valve 2 = Position 4 |
| 3.69 | Valve Position | Valve 6 = Position 2 |
| 3.71 | Dispenser (Dispenser 1) | Dispenser 1 Aspirates 4000 µl from 1 (4000 µl/min) |
| 4.72 | Wait for Input | Signal Dispenser 1 ready |
| 4.73 | Valve Position | Valve 6 = Position 3 |
| 4.74 | Dispenser (Dispenser 1) | Dispenser 1 Dispenses 4000 µl to 1 (4000 µl/min) |
| 5.74 | Wait for Input | Signal Dispenser 1 ready |
| 5.76 | Dispenser (Dispenser 1) | Dispenser 1 Aspirates 4000 µl from 1 (4000 µl/min) |
| 6.77 | Wait for Input | Signal Dispenser 1 ready |
| 6.78 | Dispenser (Dispenser 1) | Dispenser 1 Dispenses 4000 µl to 1 (4000 µl/min) |
| 7.79 | Wait for Input | Signal Dispenser 1 ready |
| 67.80 | Dispenser (Dispenser 1) | Dispenser 1 Aspirates 5000 µl from 1 (5000 µl/min) |
| 68.81 | Wait for Input | Signal Dispenser 1 ready |
| 68.82 | Valve Position | Valve 5 = Position 2 |
| 68.82 | Valve Position | Valve 7 = Position 2 |
| 68.82 | Valve Position | Valve 8 = Position 4 |
| 68.82 | Valve Position | Valve 9 = Position 2 |
| 68.82 | Valve Position | Valve 10 = Position 2 |
| 68.84 | Dispenser (Dispenser 1) | Dispenser 1 Dispenses 5000 µl to 1 (4000 µl/min) |
| 70.09 | Wait for Input | Signal Dispenser 1 ready |
| 70.11 | Valve Position | Valve 2 = Position 3 |
| 70.11 | Valve Position | Valve 3 = Position 3 |
| 70.11 | Valve Position | Valve 4 = Position 4 |
| 70.11 | Valve Position | Valve 6 = Position 2 |

| | | |
|---|---|---|
| 70.11 | Valve Position | Valve 7 = Position 4 |
| 71.11 | Valve Position | Valve 10 = Position 1 |
| 71.13 | Dispenser (Dispenser 1) | Dispenser 1 Aspirates 8000 µl from 1 (4000 µl/min) |
| 73.13 | Wait for Input | Signal Dispenser 1 ready |
| 73.16 | Valve Position | Valve 7 = Position 2 |
| 73.16 | Valve Position | Valve 10 = Position 2 |
| 73.18 | Dispenser (Dispenser 1) | Dispenser 1 Dispenses 8000 µl to 1 (4000 µl/min) |
| 75.18 | Wait for Input | Signal Dispenser 1 ready |
| 76.99 | Valve Position | Valve 10 = Position 1 |
| 77.00 | Stop all | - |

**References**


1. Vugts DJ. van Dongen GA. $^{89}$Zr-labeled compounds for PET imaging guided personalized therapy. Drug Discov. Today Technol.. 2011. 8. 53-61.
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3. Coenen HH. Gee AD. Adam M. Antoni G. Cutler CS. Fujibayashi Y. Jeong JM. Mach RH. Mindt TL. Pike VW. Windhorst AD. Consensus nomenclature rules for radiopharmaceutical chemistry - Setting the record straight.. Nucl. Med. Biol.. 2017. 55. v-xi