

## **Automated radiolabeling procedures for the preparation of OncoFAP-based radiopharmaceuticals for cancer imaging and therapy**

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## Chemical synthesis

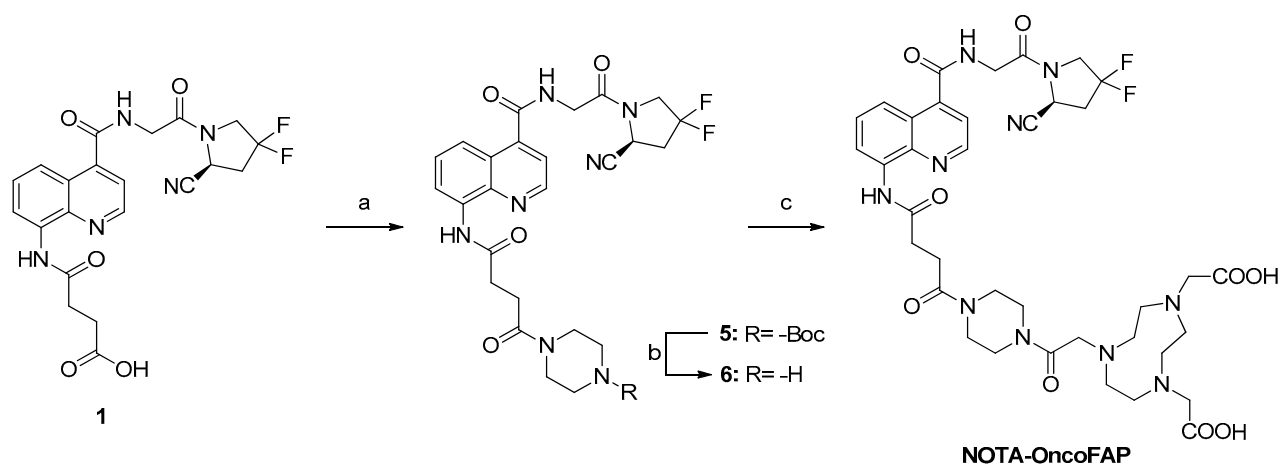
All reagents and solvents were purchased from Sigma Aldrich, VWR, CheMatech, and Combi-Blocks and used as supplied.

High Pressure Liquid Chromatography/Mass-Spectrometry (HPLC-MS) spectra presented were recorded on an Agilent 6100 Series Single Quadrupole MS system combined with an Agilent 1200 Series LC, using an InfinityLab Poroshell 120 (EC-C18 column, 4.6 × 50 mm, particle size: 2.7 µm, pore size: 120 Å) at a flow rate of 0.8 mL/ min.

Time (min)	Water + 0.1% HCOOH	ACN + 0.1% HCOOH	Flow (mL/min)
0.00	90.0 %	10 %	0.800
0.50	90.0 %	10 %	0.800
3.00	0.0 %	100 %	0.800
3.50	0.0 %	100%	0.800

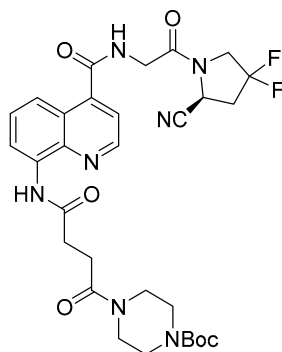
Reversed-phase high-pressure liquid chromatography (RP-HPLC) were performed on an Agilent 1200 Series RP-HPLC with PDA UV detector, using a Synergi MAX-RP (C18 column, 10 × 250 mm, particle size: 10 µm, pore size: 80 Å) at a flow rate of 5 mL/min with linear gradients of solvents A and B (A = Millipore water with 0.1% TFA, B = ACN with 0.1% TFA).

## General synthetic scheme of NOTA-OncoFAP



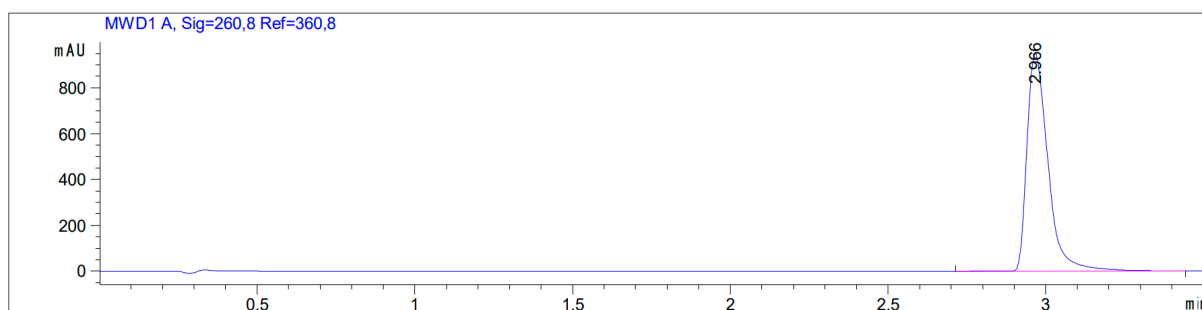
a) *N*-Boc-piperazine, HATU, DIPEA, DMF, r.t., 30 min; b) TFA/DCM 30% v/v, r.t., 3h; c) NOTA-NHS, DIPEA, DMF, r.t., 1h.

Synthesis of tert-butyl (S)-4-(4-((4-((2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-8-yl)amino)-4-oxobutanoyl)piperazine-1-carboxylate (**5**)

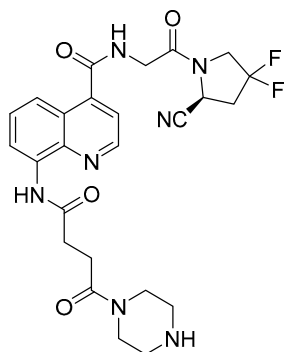


(S)-4-((4-((2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-8-yl)amino)-4-oxobutanoic acid (compound **1**) (50 mg, 0.11 mmol, 1 eq), *N*-Boc-piperazine (24 mg, 0.13 mmol, 1.2 eq) and HATU (49 mg, 0.13 mmol, 1.2 eq) were dissolved in DMF (1 mL). DIPEA (0.08 mL, 0.44 mmol, 4 eq) was added dropwise and the mixture was stirred for 30 min at room temperature. The mixture was purified via RP flash chromatography (Büchi Sepacore 40  $\mu$ m irregular column) using a gradient of 2:98 to 100:0 ACN + 0.1% HCOOH/water + 0.1% HCOOH in 40 min. The desired fractions were collected and lyophilized to afford a white solid. (40 mg, 58%)

MS (ESI+)  $m/z$  627.9 [M+H]

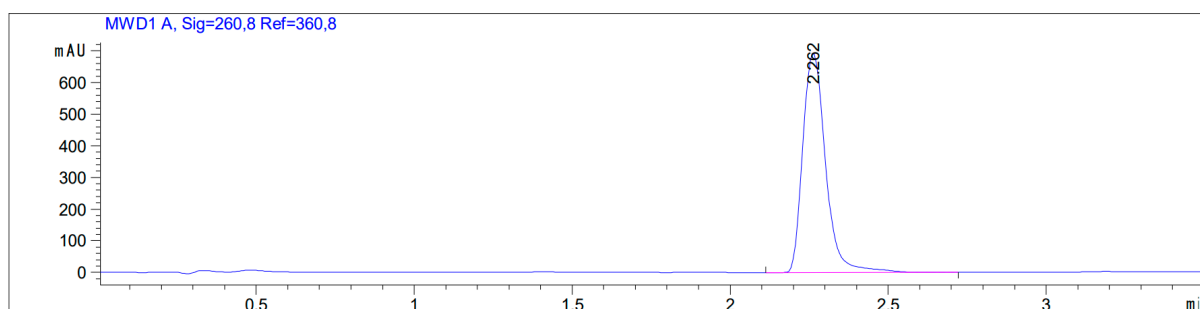


*Synthesis of (S)-N-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)-8-(4-oxo-4-(piperazin-1-yl)butanamido)quinoline-4-carboxamide (6)*

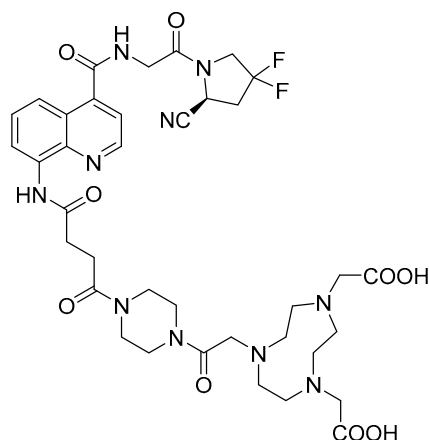


Compound **5** (40 mg, 0,06 mmol, 1 eq.) was dissolved in DCM (1 mL) and TFA (0.5 mL) was added dropwise. The mixture was stirred at room temperature for 3h then solvent was evaporated, and the crude was purified via RP flash chromatography (Büchi Sepacore 40  $\mu$ m irregular column) using a gradient of 2:98 to 100:0 ACN + 0.1% HCOOH/water + 0.1% HCOOH in 40 min. The desired fractions were collected and lyophilized to afford a colorless oil. (17 mg, 51%)

MS (ESI+)  $m/z$  527.9  $[M+H]^+$

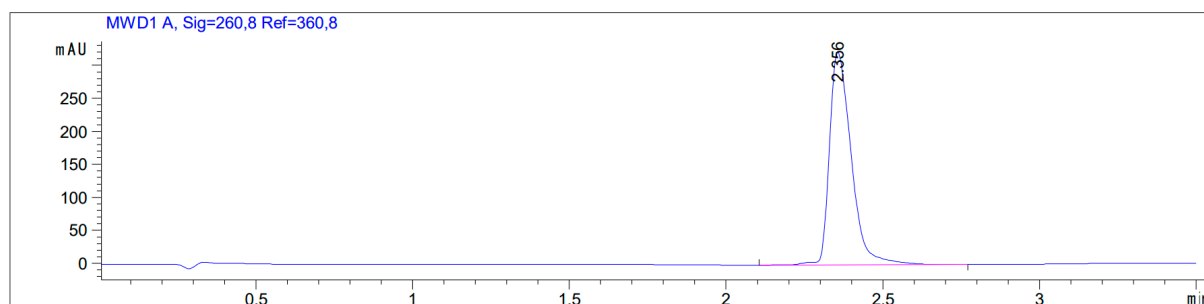


Synthesis of (S)-2,2'-(7-(2-(4-(4-((4-((2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-8-yl)amino)-4-oxobutanoyl)piperazin-1-yl)-2-oxoethyl)-1,4,7-triazonane-1,4-diyl)diacetic acid, **NOTA-OncoFAP (4)**

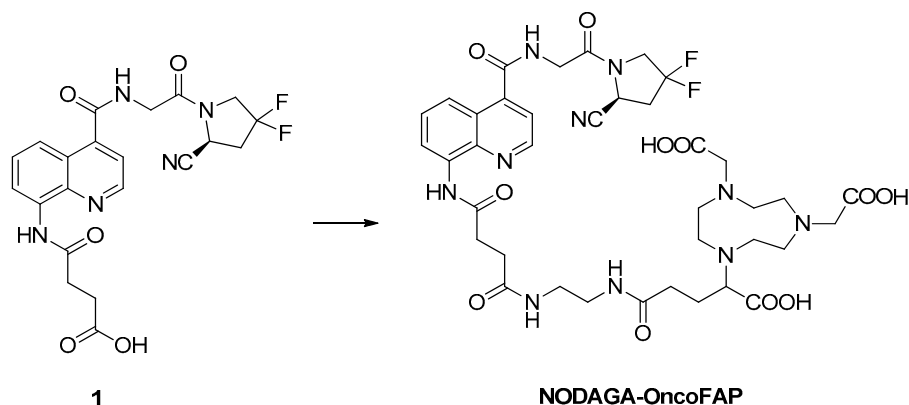


Compound **6** (8 mg, 0.02 mmol, 1 eq) and NOTA-NHS (19 mg, 0.04 mmol, 2 eq) were dissolved in DMF (0.2 mL). DIPEA (0.01 mL, 0.08 mmol, 4 eq) was added to the mixture and stirred for 1h at room temperature. The mixture was purified via RP-HPLC (90:10 to 0:100 water + 0.1% TFA/ACN + 0.1% TFA in 12 min). The desired fractions were collected and lyophilized to afford a white solid. (4 mg, 25%)

MS (ESI+)  $m/z$  812.7  $[M+H]^+$

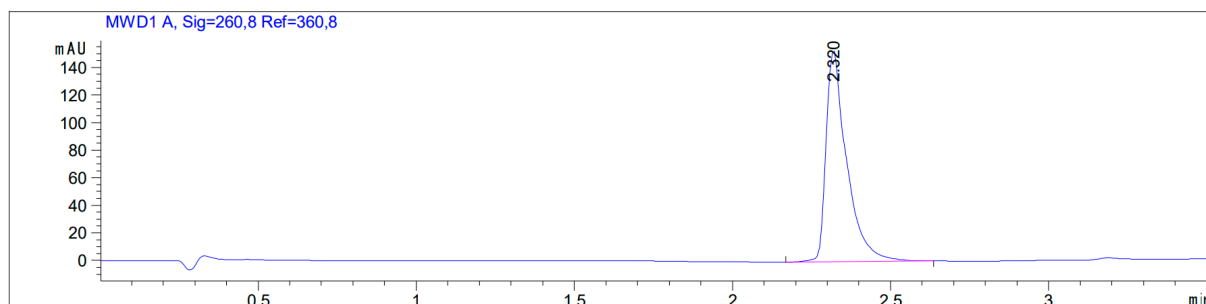


### General scheme and synthesis of OncoFAP-NODAGA (3)

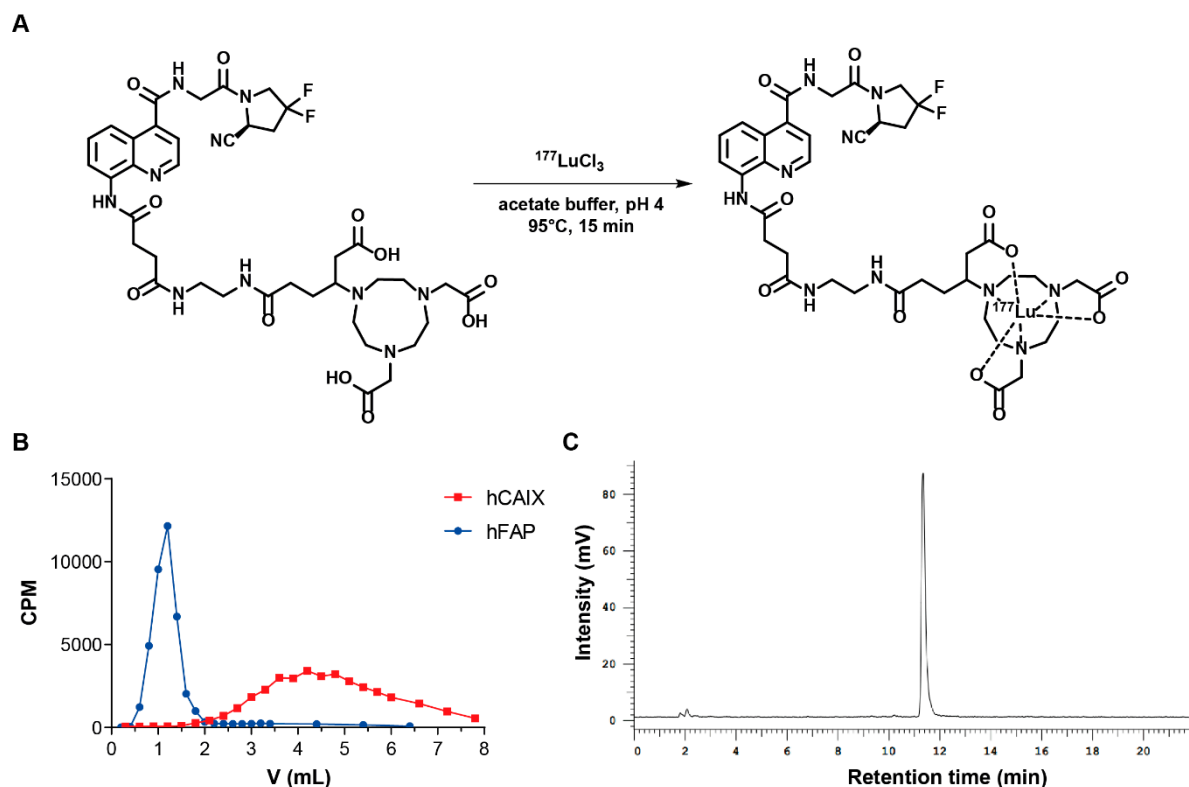


To a solution of compound **1** (50 mg, 0.11 mmol, 1 eq) in dry DMSO (1 mL) were added *N*-hydroxy succinimide (19 mg, 0.16 mmol, 1.5 eq), HATU (61 mg, 0.16 mmol, 1.5 eq) and DIPEA (57  $\mu$ L, 0.44 mmol, 4 eq), the mixture at room temperature. After 30 min NODA-GA-NH<sub>2</sub> (46 mg, 0.22 mmol, 2 eq) and water (0.3 mL) were added, and the mixture was stirred for 2h. The reaction mixture was directly purified via RP-HPLC (90:10 to 0:100 water + 0.1% TFA/ACN + 0.1% TFA in 12 min). The desired fractions were collected and lyophilized to afford a white solid. (30 mg, 32%)

MS (ESI+)  $m/z$  859.4  $[M+H]^+$



## NODAGA-OncoFAP labeling with $^{177}\text{Lu}$ and characterization

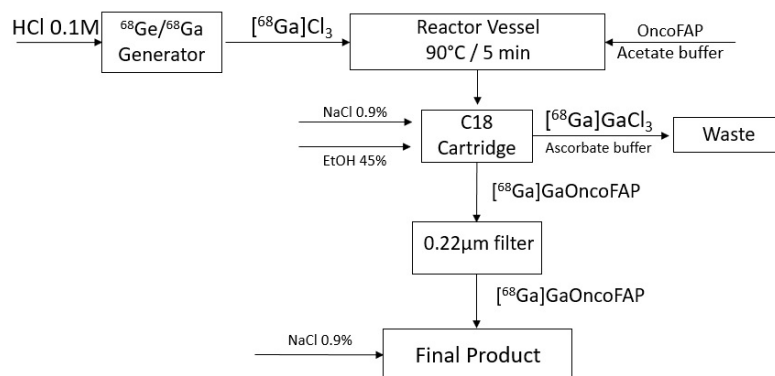


**Figure S1.** (A) Radiolabeling reaction scheme of OncoFAP-NODAGA with  $^{177}\text{Lu}$  in acetate buffer (pH=4) at 95°C for 15 min. (B) Coelution experiment of [ $^{177}\text{Lu}$ ]Lu-NODAGA-OncoFAP with hFAP and hCAIX on PD-10 size exclusion chromatography columns. (C) Radio-HPLC analysis performed after labeling reaction.

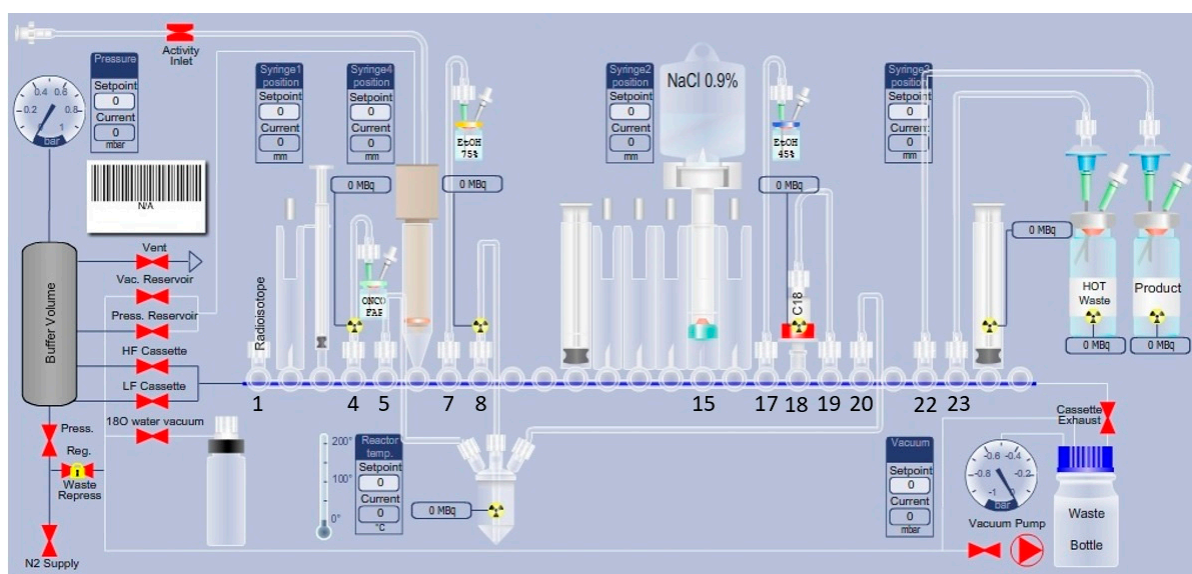
NODAGA-OncoFAP can be efficiently labeled with  $^{177}\text{Lu}$  by heating at 95°C in acetate buffer (pH=4) for 15 min. [Figure S1A] After labeling with 1 MBq activity the Radio-HPLC trace shows complete incorporation of  $^{177}\text{Lu}$  and a single peak profile (no product degradation observed) [Figure S1B]. The coelution experiment confirms the strong affinity of [ $^{177}\text{Lu}$ ]Lu-NODAGA-OncoFAP for hFAP, while no significant interaction with hCAIX, as a non-target protein, has been observed. [Figure S1C]



## Radiosynthesis of [ $^{68}\text{Ga}$ ]Ga-OncoFAP

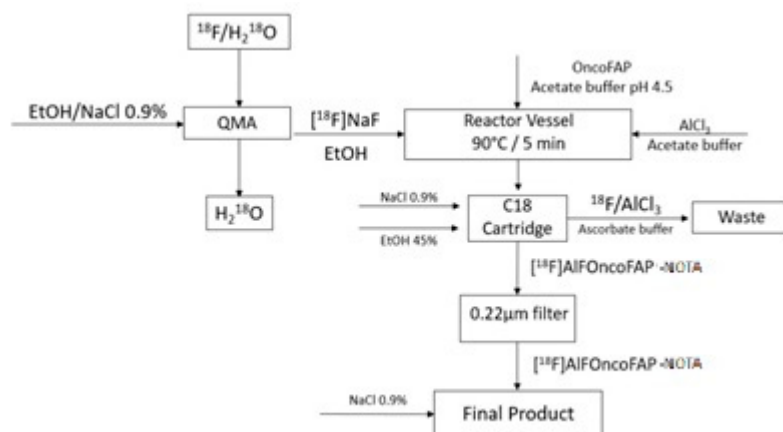


**Figure S2.** flow chart for radiosynthesis of [ $^{68}\text{Ga}$ ]Ga-OncoFAP

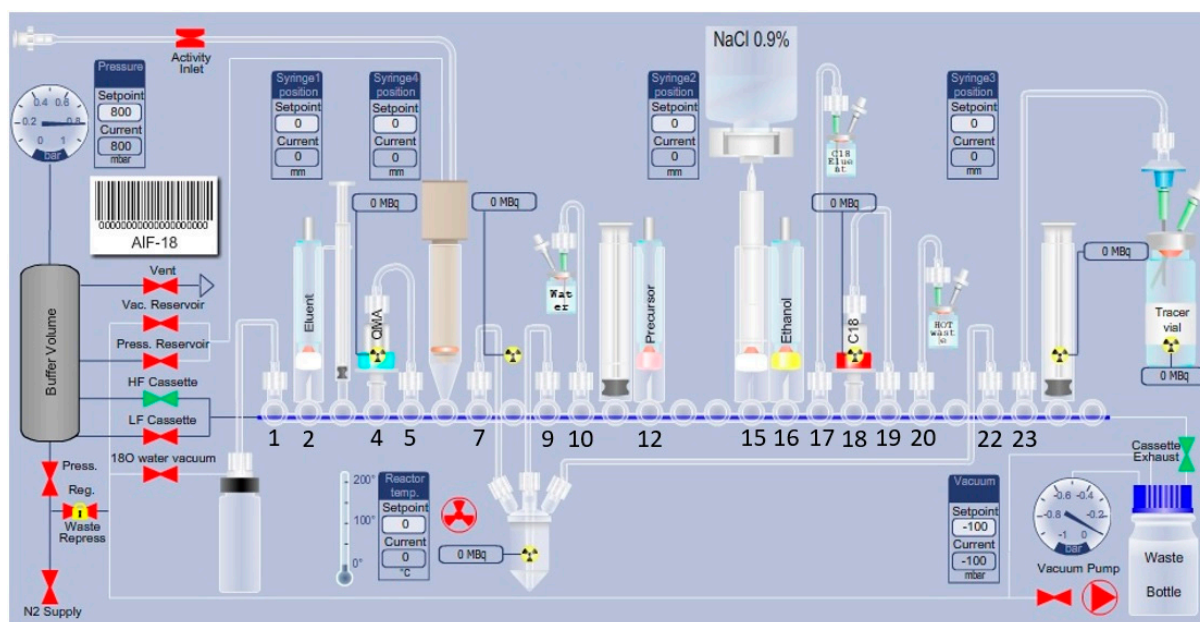


**Figure S3.** Layout of the cassette for radiosynthesis of [ $^{68}\text{Ga}$ ]Ga-OncoFAP derivatives. Position: 1) Generator; 4) Vial containing precursor dissolved in 0.7 M sodium acetate solution (650 µL); 5, 8, 20) Reactor; 7) Awakening solution (75% EtOH); 15) 0.9% NaCl solution; 17) Eluent solution (45% EtOH); 18, 19) C18 cartridge; 22) Product vial; 23) Hot waste.

## Radiosynthesis of [ $^{18}\text{F}$ ]AlF-ONcoFAP



**Figure S4.** Flow chart for radiosynthesis of [ $^{18}\text{F}$ ]AlF-ONcoFAP



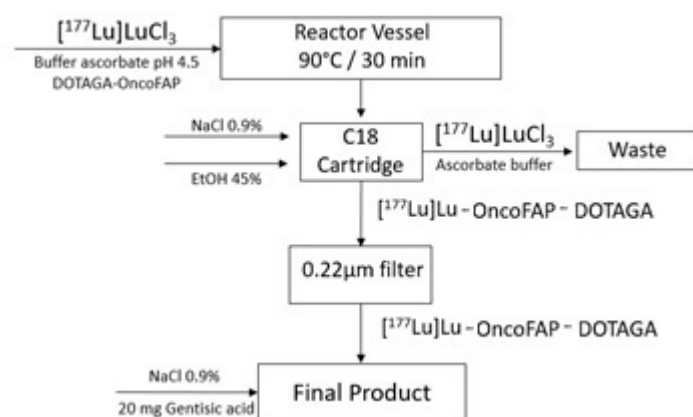
**Figure S5.** Layout of the cassette for radiosynthesis of [ $^{18}\text{F}$ ]AlF-ONcoFAP-; 1) Recovery  $\text{H}_2^{18}\text{O}$ ; 2) QMA Eluent; 4,5) QMA cartridge; 6) Conical vial connected to vial with  $^{18}\text{F}$ ; 7, 9, 22) Reactor; 10) OncoFAP-NTA solution dissolved in acetate buffer pH 4.1; 15) 0.9% NaCl solution; 16) Awakening solution (75% EtOH); 17) Eluent solution (45% EtOH); 18-19) C18 cartridge; 20) Hot Waste; 22) Product Vial.

## HPLC purification

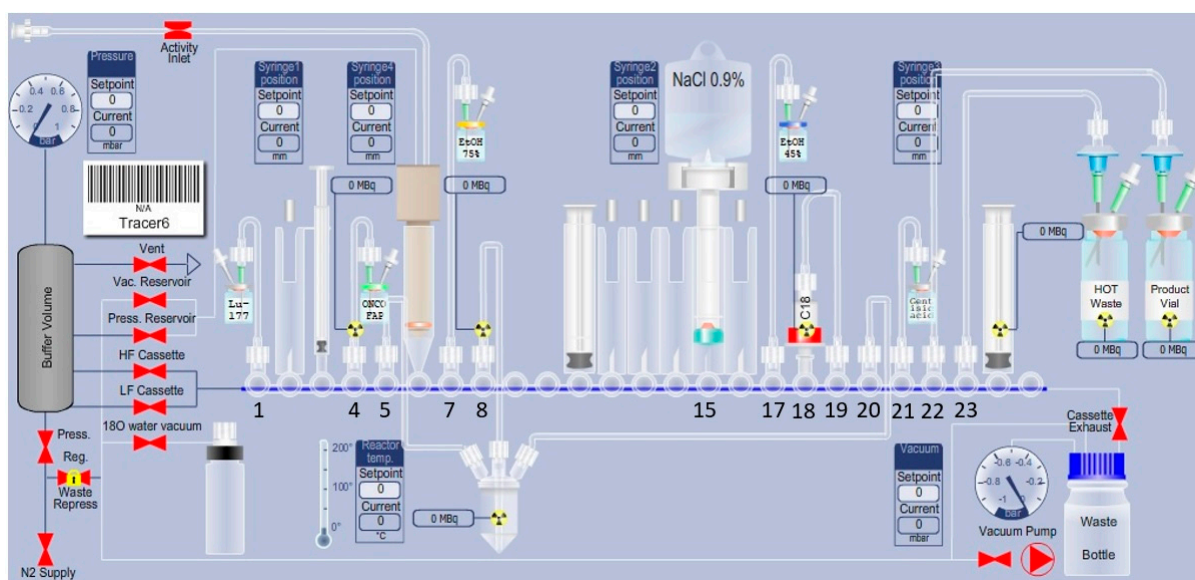
Fluorine-18 radiolabelling of NODAGA-ONcoFAP was achieved as follow: [ $^{18}\text{F}$ ]Fluorine (647 MBq) was trapped in the cartridge (45mg PS- $\text{HCO}_3^-$ ) and washed with 3 mL metal-free  $\text{H}_2\text{O}$  and dried with 7 mL air. The cartridge was then eluted with 100  $\mu\text{L}$  of 0.9% NaCl solution directly in the  $\text{AlCl}_3$  solution (2 mM; pH 4.7; 10  $\mu\text{L}$ ) and reacted for 5 min. 10  $\mu\text{L}$  of OncoFAP-NODAGA in sodium acetate (0.5 M, pH 4, 50  $\mu\text{g}$ ) and 200  $\mu\text{L}$  of ACN was added to the solution and the resulting reaction mixture was heated at 100°C for 20 min. After the labelling, the

mixture was injected on HPLC (Synergy 4  $\mu\text{m}$  Hydro-RP 80Å; 20/80 ACN/water TFA 0.1% gradient 30min). The product peak was collected at 5.8 min and diluted in 60  $\mu\text{L}$  of water. The solution passed through an Oasis® HLB 1 cc/30 mg and the product was eluted with 400  $\mu\text{L}$  ethanol and reconstituted with 0.9% NaCl solution (5 mL). The RCY (decay corrected) of this reaction is 3% and the RCP >95%.

## Radiosynthesis of [ $^{177}\text{Lu}$ ]Lu-DOTAGA-OncoFAP

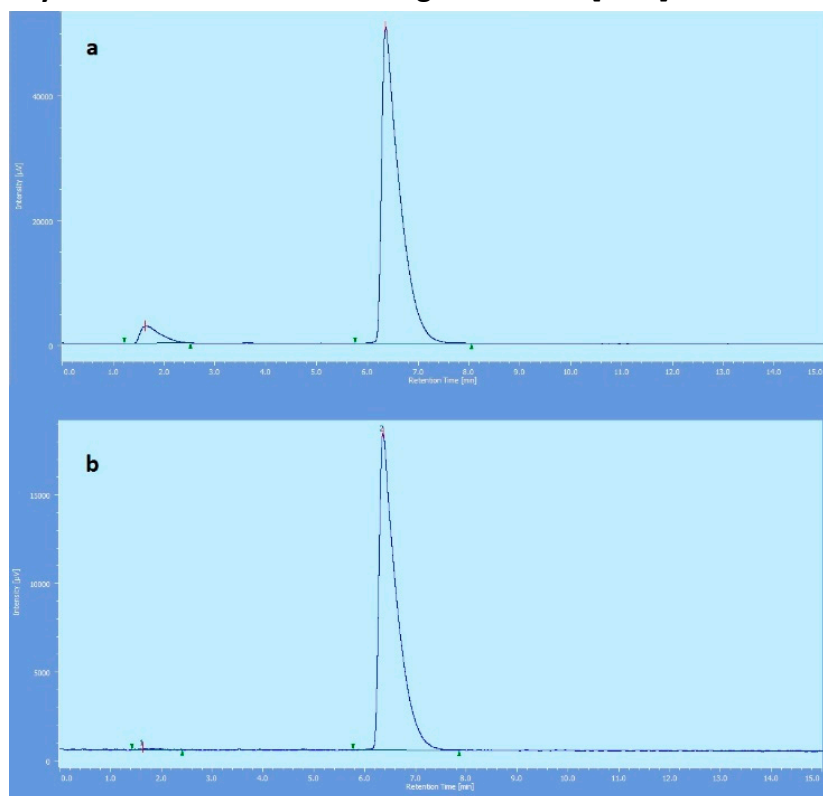


**Figure S6.** Flow chart for radiosynthesis of [ $^{177}\text{Lu}$ ]Lu-DOTAGA-OncoFAP



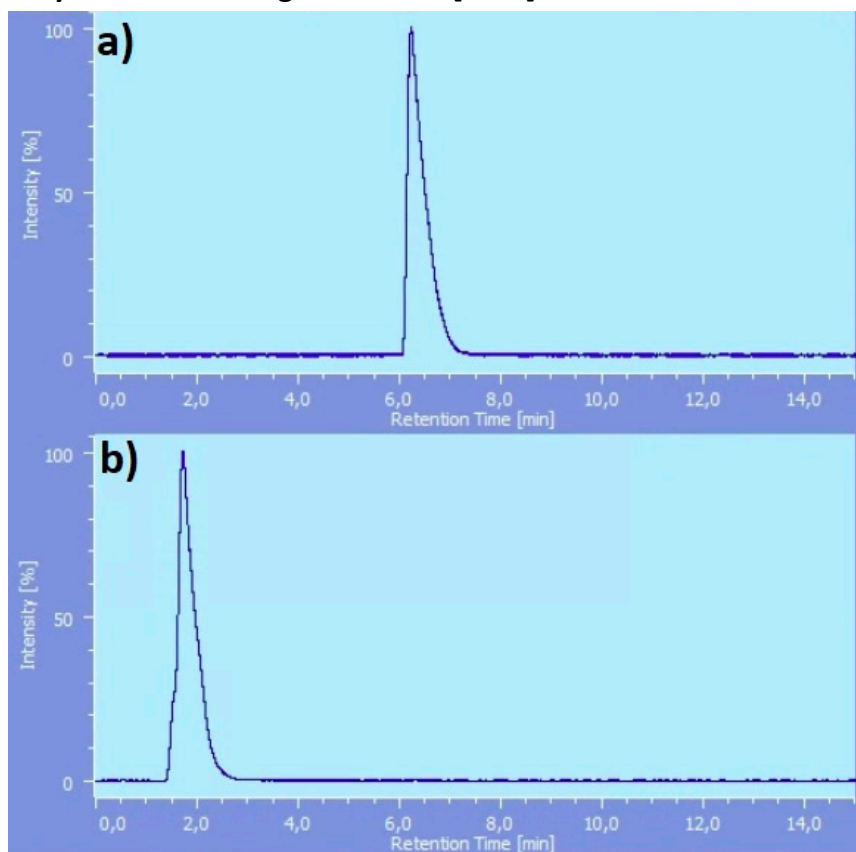
**Figure S7.** Layout of the cassette for radiosynthesis of [ $^{177}\text{Lu}$ ]Lu-DOTAGA-OncoFAP; Position: 1). Vial of  $^{177}\text{Lu}$  in acetate buffer pH 4.5; 4) DOTAGA-OncoFAP in acetate buffer pH 4.5; 5, 8, 20) Reactor; 7) Awakening solution (75% EtOH); 15) 0.9% NaCl solution; 16) Awakening solution (75% EtOH); 17) Eluent solution (45% EtOH); 18,19) C18 cartridge; 21) Gentisic Acid dissolved in 0.9% NaCl solution; 22) Product vial; 23) Hot Waste.

**Radio-HPLC analysis before and after labelling reaction of [ $^{68}\text{Ga}$ ]Ga-DOTAGA-OncoFAP**



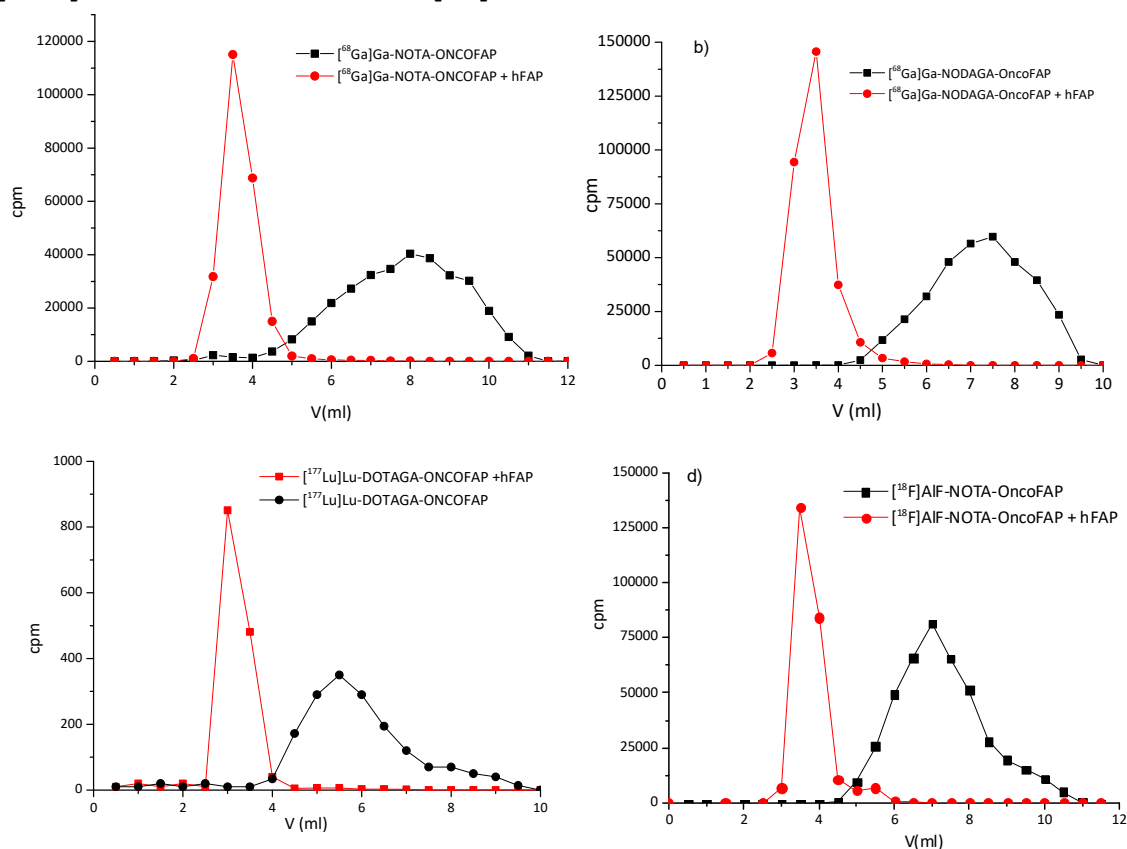
**Figure S8.** Radio-HPLC trace performed after radiosynthesis of [ $^{68}\text{Ga}$ ]Ga-DOTAGA-OncoFAP (A) before purification with C18 cartridge and (B) after purification with C18.

**Radio-HPLC analysis after labelling reaction of [ $^{177}\text{Lu}$ ]Lu-DOTAGA-OncoFAP**



**Figure S9.** Radio-HPLC trace performed after radiosynthesis of [ $^{177}\text{Lu}$ ]Lu-DOTAGA-OncoFAP (A) product (B) "Hot" waste of the synthesis

**Co-elution experiments of [<sup>68</sup>Ga]Ga-NOTA-OncoFAP; [<sup>68</sup>Ga]Ga-NODAGA-OncoFAP, [<sup>177</sup>Lu]Lu-DOTAGA-OncoFAP and [<sup>18</sup>F]AlF-NOTA-OncoFAP with hFAP**



**Figure S10.** Co-elution experiment of radiolabelled preparations of (A) [<sup>68</sup>Ga]Ga-NOTA-OncoFAP; (B) [<sup>68</sup>Ga]Ga-NODAGA-OncoFAP (C) [<sup>177</sup>Lu]Lu-DOTAGA-OncoFAP (D) [<sup>18</sup>F]AlF-NOTA-OncoFAP with hFAP and without hFAP on PD-10 size exclusion chromatography columns.

**Radiolabeling Simple kit**

In all synthesis we used a reaction time of 10 minutes, a reaction temperature of 95°C and we have obtained a final volume of 4.5ml.

Radiosynthesis [ <sup>68</sup> Ga]Ga-NODAGA-OncoFAP			
Formate buffer (Simple Preparation)			
	Batch 1	Batch 2	Batch 3
<b>NODAGA-OncoFAP (μg)</b>	10	20	40
<b>Starting activity (GBq)</b>	1.11±0.3	1.13±0.3	1.05±0.3
- Free gallium-68 content	6.2±0.3%	3.0±0.3%	1.3±0.3%
- Colloidal <sup>68</sup> Ga content	1.3±0.3%	1.7±0.3%	1.5±0.3%
- Radiochemical purity	92.5±0.3%	95.3±0.3%	97.2±0.3%

**Table S1.** Different conditions used for the synthesis [<sup>68</sup>Ga]Ga-NODAGA-OncoFAP

Radiosynthesis [ <sup>68</sup> Ga]Ga-NODAGA-OncoFAP Acetate buffer (Simple Preparation)			
	Batch 1	Batch 2	Batch 3
NODAGA-OncoFAP (μg)	10	20	40
Starting activity (GBq)	1.15±0.3	0.98±0.3	1.12±0.3
- Free gallium-68 content	14.2±0.3%	10.3±0.3%	6.3±0.3%
- Colloidal <sup>68</sup> Ga content	7.5±0.3%	7.4±0.3%	6.5±0.3%
- Radiochemical purity	78.3±0.3%	82.3±0.3%	87.2±0.3%

**Table S2.** Different conditions used for the synthesis [<sup>68</sup>Ga]Ga-OncoFAP-NODAGA

Radiosynthesis [ <sup>68</sup> Ga]Ga-DOTAGA-OncoFAP Formate buffer (Simple Preparation)			
	Batch 1	Batch 2	Batch 3
DOTAGA-OncoFAP	10	20	40
Starting activity (GBq)	1.04±0.3	0.97±0.3	0.93±0.3
- Free gallium-68 content	12.9±0.3%	4.2±0.3%	3.3±0.3%
- Colloidal <sup>68</sup> Ga content	1.9±0.3%	1.7±0.3%	1.7±0.3%
- Radiochemical purity	85.2±0.3%	94.1±0.3%	95.0±0.3%

**Table S3.** Different conditions used for the synthesis [<sup>68</sup>Ga]Ga-OncoFAP-NODAGA

Radiosynthesis [ <sup>68</sup> Ga]Ga-DOTAGA-OncoFAP Acetate buffer (Simple Preparation)			
	Batch 1	Batch 2	Batch 3
DOTAGA-OncoFAP	10	20	40
Starting activity (GBq)	1.08±0.3	1.02±0.3	0.95±0.3
- Free gallium-68 content	14.7±0.3%	9.8%	6.6%
- Colloidal <sup>68</sup> Ga content	9.0%	8.7%	7.1%
- Radiochemical purity	76.3%	81.5%	86.3%

**Table S4.** Different conditions used for the synthesis [<sup>68</sup>Ga]Ga-OncoFAP-NODAGA

#### Assessment of *in vitro* lipophilicity

Product	LogP	LogD <sub>7.4</sub>
[ <sup>68</sup> Ga]Ga-NOTA-OncoFAP	-2.38± 0.11	-2.19± 0.11
[ <sup>68</sup> Ga]Ga-NODAGA-OncoFAP	-2.68± 0.14	-2.48± 0.09
[ <sup>68</sup> Ga]Ga-DOTAGA-OncoFAP	-2.57± 0.15	-2.39 ± 0.12
[ <sup>18</sup> F]AIF-NOTA-OncoFAP	-2.15± 0.18	-2.01± 0.25
[ <sup>18</sup> F]AIF-NODAGA-OncoFAP	-3.02± 0.15	-2.88±0.16
[ <sup>177</sup> Lu]Lu-DOTAGA-OncoFAP	-2.01± 0.08	-1.85± 0.05

**Table S5.** LogP and LogD<sub>7.4</sub>