# Development, Validation, and Concordance of Two MAGE-A4 Immunohistochemistry (IHC) Assays to Establish Prognostic Value of MAGE-A4 Expression in Synovial Sarcoma

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## Introduction

- The investigational therapy afamitresgene autoleucel (afami-cel) is a human leukocyte antigen–restricted autologous T-cell receptor T-cell therapy that targets solid tumors expressing melanoma-associated antigen A4 (MAGE-A4)
- Serving as a patient eligibility criterium, MAGE-A4 antigen positivity is determined with a validated laboratory-developed test at central CellCarta laboratories located in Antwerp, Belgium for European patients, or Naperville, IL, USA for North American patients
- Promising clinical responses in patients with MAGE-A4–expressing synovial sarcoma (SyS) have been observed in single-arm Phase 1 and 2 clinical trials of afami-cel[1,2]
- However, these single-arm clinical trials necessitate the investigation of the potential bias introduced by these biomarkers in regard to the clinical outcomes of the study population compared with the overall patient population
- SyS is a rare disease, making it challenging to identify large data sets of clinical cases paired with samples for biomarker testing
- One such large medical database paired with biopsies from patients with SyS exists at the Centre de Lutte contre le cancer Léon Bérard (CLB)

## Objective

- To develop a solution allowing uniform MAGE-A4 expression analysis within clinical trials and in prognostic studies to assess the impact of MAGE-A4 expression in SyS
  - We describe the validation at CellCarta, Antwerp, Belgium, of an immunohistochemical (IHC) clinical trial assay (CTA) on the Dako Link 48 autostainer measuring MAGE-A4 expression
  - Because CLB uses the Ventana BenchMark ULTRA autostainer, and samples from CLB cannot be tested outside France due to legal restrictions, we therefore describe the development at CLB of a comparable IHC assay to closely mimic the CTA, and thus be an equivalent IHC assay to determine MAGE-A4 expression in archival samples

## CellCarta assay validation of MAGE-A4 CTA to serve as a laboratory-developed test for patient enrollment in Adaptimmune clinical trials

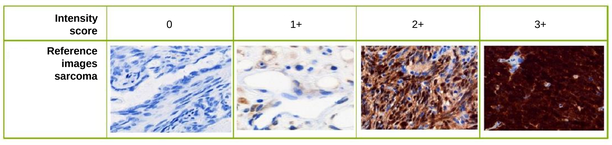
- The MAGE-A4 CTA was developed by CellCarta on the Dako Link 48 autostainer platform using the anti–MAGE-A4 mouse monoclonal antibody (clone ID: OTI1F9, lot F002, OriGene) to measure MAGE-A4 expression levels in formalin-fixed paraffin-embedded (FFPE) tissue samples
- Scoring methods for SyS FFPE samples are shown in **Table 1** and **Figure 1**
- All MAGE-A4 scoring occurred at a central laboratory (CellCarta) by trained pathologists
- MAGE-A4 positivity was defined as ≥30% of a sample's tumor cells showing MAGE-A4 positivity at ≥2+ intensity
- Validation of the CTA was composed of prevalence, precision, and pathologist concordance (using a subset of prevalence samples) assays, followed by assay transfer tests between EU and US CellCarta laboratories, and US/EU pathologist concordance testing
- Acceptance criteria for precision validation were:
  1. Precision is assessed qualitatively. The staining pattern of serial sections must be ≥80% concordant visually. All negative cases must be negative in all runs. All positive cases must be positive in all runs. All weak cases must be weak in all runs. All strong cases must be strong in all runs
  2. All serial sections should be MAGE-A4 positive (ie, ≥30% of tumor cells with MAGE-A4 positivity at ≥2+ intensity) or negative. Results must be ≥80% concordant (ie, ≥80% over the different samples and within one sample)
  3. Samples not meeting the approval criteria for positivity will be tested for a second concordance criterion: the %CV for each sample should not exceed 20% and results must be ≥80% concordant (ie, over the different samples and within one sample, scores fall within the %CV acceptance criteria)
- Assay transfer and pathologist concordance acceptance criterion were the same as above. The US/EU pathologist concordance required ≥85% concordance between pathologist and consensus score

## Table 1. Scoring method for MAGE-A4 expression

| Slides | Pathologist assessment |
|---|---|
| Hematoxylin & eosin | Confirm that tumor is present and fulfills prerequisites for scoring |
| Mouse IgG negative control | Determine the level of background staining, non-specific staining, and artefacts |
| MAGE-A4 | • Determine the number of tumor cells with nuclear or cytoplasmic MAGE-A4 expression at 0, 1+, 2+, and 3+ intensity<br>• Report the score at each intensity as a percentage of MAGE-A4–positive tumor cells relative to the total number of cells |

Ig, immunoglobulin; MAGE-A4, melanoma-associated antigen A4.

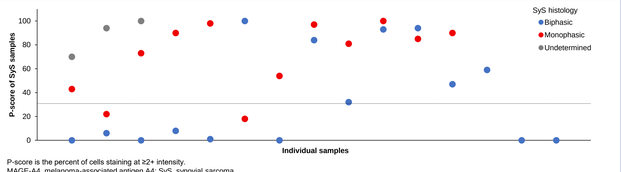
## Figure 1. Reference images for MAGE-A4 intensity




MAGE-A4, melanoma-associated antigen A4.

- Prevalence of MAGE-A4 expression in SyS tumor samples was determined by staining and scoring 30 samples (**Figure 2**)

## Figure 2. Distribution of MAGE-A4 P-score in prevalence study SyS samples

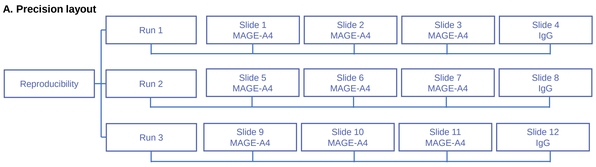



P-score is the percent of cells staining at ≥2+ intensity.
MAGE-A4, melanoma-associated antigen A4; SyS, synovial sarcoma.

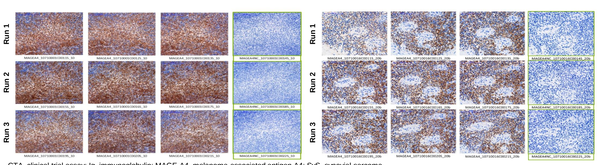
- In a precision study, the CTA passed acceptance criteria and demonstrated high reproducibility (**Figure 3**, **Table 2**)
  - The precision study was performed on five SyS samples selected based on the dynamic range of MAGE-A4 expression seen in the prevalence study
  - The prevalence study indicated these five samples had MAGE-A4 expression levels of 0%, 32%, 43%, 47%, and 100% of cells staining with ≥2+ intensity (**Figure 2**)
  - In the precision study, 100% agreement was observed for positive/negative agreement in intra-run and inter-run concordance
  - All precision samples met acceptance criteria and were concordant
  - The pathologist concordance test on 15 samples passed 100% intra-reader with an intraclass correlation coefficient (ICC) of 0.99 (95% CI: 0.98–1.00), and inter-reader variability concordance of 93%, with an ICC of 0.96 (95% CI: 0.90–0.99)
- In the assay transfer study, the CTA passed acceptance criteria, allowing for MAGE-A4 staining and scoring at both US and EU CellCarta testing locations
  - Assay transfer of 12 samples met acceptance criteria and results were concordant between US/EU laboratories
  - The US/EU pathologist concordance scoring of 29 samples also met acceptance criteria. Both US pathologists reached >85% correlation to EU pathologist scores with, an ICC of >0.7

## Figure 3. CTA precision study

**A. Precision layout**




**B. Representative images of precision study intra- and inter-run variability for two SyS samples**




CTA, clinical trial assay; Ig, immunoglobulin; MAGE-A4, melanoma-associated antigen A4; SyS, synovial sarcoma.

## Table 2. CTA precision results

| | Negative percent agreement | Positive percent agreement | Overall percent agreement | Total negative | False positive | Total positive | False negative |
|---|---|---|---|---|---|---|---|
| Intra-run | 100% | 100% | 100% | 9 | 0 | 36 | 0 |
| Inter-run | 100% | 100% | 100% | 9 | 0 | 36 | 0 |

CTA, clinical trial assay.

## Development of CLB MAGE-A4 IHC assay

- The CTA was transferred to and modified by CLB (**Table 3**, **Table 4**, **Figure 4**)
- The CLB assay was developed on a Ventana BenchMark ULTRA autostainer using the same anti–MAGE-A4 antibody
- Six protocols with six different anti–MAGE-A4 antibody dilutions were tested; the backbone of each protocol was similar and included:
  - Unmasking: Tris-EDTA pH 7.8 buffer, 32-minute incubation at 95°C
  - MAGE-A4 antibody: 32-minute incubation at 37°C
    - 1/50, 1/100, 1/200, 1/500, 1/1000, and 1/2000 dilutions
  - Detection: UltraView Universal DAB Detection Kit
- The following controls were used:
  - Normal testis: positive control
  - Normal kidney: negative control
  - Me275 melanoma cells: positive control
  - A549 non-small cell lung cancer (NSCLC) cells: negative control
  - SyS sample #1 from CLB
  - SyS sample #2 from CLB
- Assay transfer and modification testing of these six samples met acceptance criteria for inter-lab comparison by having equivalent staining pattern intensity for all samples with the CLB assay compared with those stained by the CTA

## Table 3. CTA transfer activities to CLB

| Task | Lead party |
|---|---|
| **Validation study** | |
| Adaptation of the CellCarta protocol to the Ventana stainer<br>Scanning of control samples and transfer to CellCarta<br>Transfer of scans to CellCarta | CLB |
| Staining of CLB control slides<br>Review and go decision by pathologist | CellCarta |
| **Concordance study** | |
| Staining of the concordance study cohort<br>Scanning of slides and transfer to CellCarta | CLB |
| Staining of concordance study cohort<br>Review and go decision by pathologist | CellCarta |

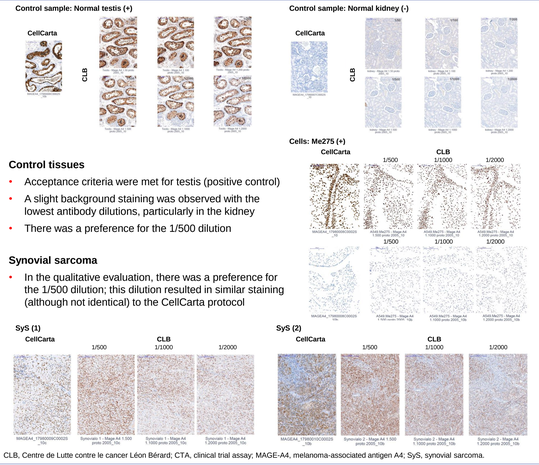
CLB, Centre de Lutte contre le cancer Léon Bérard; CTA, clinical trial assay.

## Table 4. Comparison of CLB assay and CellCarta CTA

| | CellCarta MAGE-A4 validation in SyS | CellCarta assay transfer | CLB MAGE-A4 IHC validation in SyS |
|---|---|---|---|
| Instrument | Dako Link 48 | Dako Link 48 | Ventana BenchMark ULTRA |
| Screening/ assay transfer | Screening: 30 SyS and control tissues/cell lines | Validation study: Two SyS, one normal testis (+), one normal kidney (-), one Me275 melanoma cell pellet (+), one A549 NSCLC cell pellet (-) | Validation study: Two SyS, one normal testis (+), one normal kidney (-), one Me275 melanoma cell pellet (+), one A549 NSCLC cell pellet (-) |
| Acceptance criteria | Report as found | CellCarta pathologist confirmation of same staining pattern and appropriate positive and negative control results. Go/no go decision based on inter-lab comparison | |
| Concordance samples | N/A | 5 SyS samples (and four control samples) stained on each platform:<br>1) Q3205-1: Monophasic synovial sarcoma, retropharyngeal<br>2) Q9399: Synovial sarcoma, biphasic<br>3) Q9405: Synovial sarcoma, biphasic, predominantly spindle cell component<br>4) Q9409: Synovial sarcoma, monophasic, possible met to adrenal<br>5) Q9410: Synovial sarcoma, monophasic | |
| Concordance acceptance criteria | N/A | Positive percent agreement, negative percent agreement, and overall percent agreement between the two IHC assays (CLB, CTA) shall be ≥90% | Comparative method: Test method (+ / -): a, b, c, d; Total: (a+b), (c+d)<br>Positive agreement = $\frac{a}{a+c}$ Negative agreement = $\frac{d}{c+d}$ |
| Precision | Five SyS samples (intra, inter) | N/A | Six samples (intra- and inter-run) |

CLB, Centre de Lutte contre le cancer Léon Bérard; CTA, clinical trial assay; IHC, immunohistochemical; MAGE-A4, melanoma-associated antigen A4; N/A, not applicable; NSCLC, non-small cell lung cancer; SyS, synovial sarcoma.

## Figure 4. Representative images from CLB assay development




**Control tissues**

- Acceptance criteria were met for testis (positive control)
- A slight background staining was observed with the lowest antibody dilutions, particularly in the kidney
- There was a preference for the 1/500 dilution

**Synovial sarcoma**

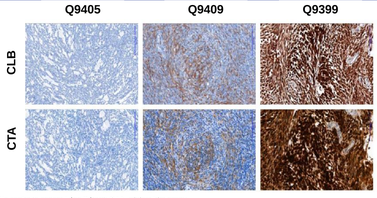
- In the qualitative evaluation, there was a preference for the 1/500 dilution; this dilution resulted in similar staining (although not identical) to the CellCarta protocol

CLB, Centre de Lutte contre le cancer Léon Bérard; CTA, clinical trial assay; MAGE-A4, melanoma-associated antigen A4; SyS, synovial sarcoma.

## Concordance was observed between the CellCarta MAGE-A4 CTA and CLB MAGE-A4 IHC assays

- The concordance study comprised five SyS samples whose MAGE-A4 status was the same in both assays, despite some qualitative differences (**Figure 5**)
- Negative, positive, and overall percent agreement scores were all 100% (**Table 5**)

## Figure 5. Concordance results




CLB, Centre de Lutte contre le cancer Léon Bérard; CTA, clinical trial assay.

## Table 5. Concordance results

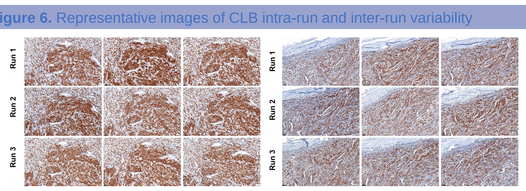
| Sample ID | Condition | 0 | 1 | 2 | 3 | % positivity at ≥2+ | Positivity status (cutoff = ≥30% at ≥2+) |
|---|---|---|---|---|---|---|---|
| 1 | TE-IHC-255 (MAGE-A4 CellCarta) | 43 | 53 | 4 | 0 | 4 | Negative |
| 1 | MAGE-A4 CLB (8-min hematoxylin) | 29 | 52 | 17 | 2 | 19 | Negative |
| 1 | MAGE-A4 CLB (12-min hematoxylin) | 24 | 52 | 21 | 3 | 24 | Negative |
| 2 | TE-IHC-255 (MAGE-A4 CellCarta) | 0 | 0 | 3 | 97 | 100 | Positive |
| 2 | MAGE-A4 CLB (8-min hematoxylin) | 0 | 0 | 7 | 93 | 100 | Positive |
| 2 | MAGE-A4 CLB (12-min hematoxylin) | 0 | 0 | 4 | 96 | 100 | Positive |
| 3 | TE-IHC-255 (MAGE-A4 CellCarta) | 97 | 2 | 1 | 0 | 1 | Negative |
| 3 | MAGE-A4 CLB (8-min hematoxylin) | 98 | 1 | 1 | 0 | 1 | Negative |
| 3 | MAGE-A4 CLB (12-min hematoxylin) | 98 | 2 | 1 | 0 | 1 | Negative |
| 4 | TE-IHC-255 (MAGE-A4 CellCarta) | 58 | 28 | 14 | 0 | 14 | Negative |
| 4 | MAGE-A4 CLB (8-min hematoxylin) | 40 | 35 | 20 | 5 | 25 | Negative |
| 4 | MAGE-A4 CLB (12-min hematoxylin) | 38 | 38 | 20 | 5 | 25 | Negative |
| 5 | TE-IHC-255 (MAGE-A4 CellCarta) | 21 | 41 | 22 | 16 | 38 | Positive |
| 5 | MAGE-A4 CLB (8-min hematoxylin) | 2 | 29 | 31 | 38 | 69 | Positive |
| 5 | MAGE-A4 CLB (12-min hematoxylin) | 1 | 34 | 29 | 36 | 65 | Positive |

CLB, Centre de Lutte contre le cancer Léon Bérard; CTA, clinical trial assay; IHC, immunohistochemical; MAGE-A4, melanoma-associated antigen A4.

## CLB inter- and intra-assay precision

- CLB intra-assay precision assay: Specimens (control tissues) demonstrating the range of MAGE-A4 expression were stained in triplicate using a single lot of antibody by a single operator on a single IHC staining run
- CLB inter-assay precision assay: Specimens demonstrating the range of MAGE-A4 expression were stained in triplicate using a single lot of antibody by a single operator on three non-consecutive days (intra-assay precision counts as Day 1 of inter-assay precision)
- A total of 54 data points were generated
- Slides from the intra- and inter-precision studies were scanned at CLB and sent to CellCarta for review and scoring
- CellCarta evaluated intra- and inter-assay precision and determined them as valid (**Figure 6** shows representative images for 2 SyS samples)

## Figure 6. Representative images of CLB intra-run and inter-run variability




CLB, Centre de Lutte contre le cancer Léon Bérard.

## Conclusions

- The MAGE-A4 CTA validated in SyS samples is highly reproducible and serves as the companion diagnostic laboratory-developed test supporting ongoing patient enrollment in the pivotal Phase 2 afami-cel trial (NCT04044768)
- The MAGE-A4 CTA was successfully adapted to be a comparable IHC assay optimized and validated at CLB, reaching 100% concordance
- The development, validation, and equivalence of the CTA and CLB assays together allow uniform assessment of MAGE-A4 expression in FFPE SyS tissue samples
- This CLB IHC assay:
  - Meets the criteria required to be used in a retrospective prognostic study at CLB, and a similar MAGE-A4 positivity status as obtained with the CTA can be expected
  - Is appropriate to evaluate the potential impact of MAGE-A4 expression on survival of patients with SyS

Presented at the 38th Annual Meeting of the Society for Immunotherapy of Cancer, San Diego, CA, USA, November 1-5, 2023
Abstract ID: 198

**References**
1. Hong DS, et al. *Nat Med.* 2023;29:104.
2. Van Tine BA, et al. Oral presented at CTOS 2022; Vancouver, BC, Canada.

**Footnotes and Abbreviations Used in Text**
afami-cel; afamitresgene autoleucel; CLB, Centre de Lutte contre le cancer Léon Bérard; CTA, clinical trial assay; CV, coefficient of variation; FFPE, formalin-fixed paraffin-embedded; ICC, intraclass correlation coefficient; IHC, immunohistochemical; MAGE-A4, melanoma-associated antigen A4; NSCLC, non-small cell lung cancer; SyS, synovial sarcoma.

**Acknowledgments and Disclosures**
This study was funded by Adaptimmune
Writing/editorial assistance was provided by Christine Ingleby, DPhil, CMPP, of Envision Pharma Group, funded by Adaptimmune