Sigma-Aldrich®
Lab & Production Materials

Application Note

Development of CLIA for the Detection of the Cardiac Troponin-I Marker

Influence of carboxyl superparamagnetic bead parking areas

INTRODUCTION

The Chemiluminescent ImmunoAssay (CLIA) is usually a heterogeneous assay format that can be used in high throughput analyzers for *in vitro* diagnostics (IVD). It offers higher analytical sensitivity than other immunoassay formats such as immunoturbidimetry or ELISA. In heterogeneous CLIA, antibodies or antigens are usually immobilized on superparamagnetic beads as the solid phase, allowing washing steps along the assay and reducing potential unspecific adsorptions. Therefore, the selection of the superparamagnetic beads to be used is a critical factor which has a direct impact on the functionality of the assay.

There are general parameters to be considered in order to select superparamagnetic beads for a chemiluminescent immunoassay:

Size: Although magnetic beads are usually homogenized and kept in suspension on board the CLIA analyzers, big beads may sediment if the shaking of the analyzer is insufficient.

Smaller beads offer higher surface-to-weight ratio available to attach proteins onto them, which can lead to increase the analytical sensitivity of the reagent; however, small beads require longer time separations.

Therefore, medium-sized beads around 1-2 µm are most often used for chemiluminescent immunoassays.

Composition and Special Properties: Beads with low content of magnetic pigment will require longer separation timings. Beads with 30-50% of magnetic pigment content are usually used.

Coating & Surface Chemistry: Plain beads are widely used to attach proteins easily by adsorption. However, this kind of protein immobilization could be weak and may lead to an unstable reagent, affecting the sensitivity and/or specificity of the reagent.

A chemically modified surface offers the chance to conjugate proteins covalently onto the beads. Carboxyl paramagnetic beads are a well-known technology that offers a robust and stable link of antibodies to the beads surface.

Superparamagnetic beads of 1 µm, with approximately 40% of magnetic pigment and modified with carboxyl functional groups were preliminarily selected for this technical note. The surface charge density of functional groups is another important parameter to be considered. The parking area (A2/group) describes how much space the functional group occupies, and it is related to the particle size, particle density, and content of functional groups present on the surface of the beads.

This technical note shows the functionality of carboxyl superparamagnetic beads with three different Parking Areas (PA) during the development of a chemiluminescent immunoassay for the quantitative detection of Troponin-I (cTnI).

Troponin is a protein complex of three subunits (T, I, and C) that is involved in the contractile process of skeletal and cardiac muscle. Both cardiac and skeletal muscle express troponin C, whereas troponin T and I are generally thought to be cardiac-specific and are used as biomarkers of choice for the diagnosis of cardiac injury[1]. However, a recent study has challenged whether troponin T is exclusively cardiac-specific[2].

MERCK

MATERIALS AND METHODS

Estapor® Carboxylated Microspheres (-COOH) Ref. EM1-100/40, Cat. No 23 710 087

- Lot M8965/5 (29 µeq/g, PA = 20)
- Lot M8972/3 (41 µeq/g, PA = 15)
- Lot M8946/4 (70 µeq/g, PA = 8)

Equipment

A magnetic Separator (SEPMAG, A200ml) was used during beads coating procedure.

A multimode plate reader (Perkin Elmer, Victor Nivo HH35000311) was used for triggers dispensation and performing measurement readings.

BioMag® 96-well plate side pull magnetic separator (85072S) was used to carry out the washing steps during the assay.

Reagents

The antibodies used were the primary monoclonal Anti-TnI capture antibody (Meridian Life Science, H86280M) and the secondary Anti cTnI-biotin antibody (Meridian Life Science, H86207B).

Streptavidin (SIGMA, S0677) was conjugated with the CLIA label Tag.

Commercial Trigger solution 1 and 2 were used (ENZO life science, ADI-906-001).

Native Troponin I standard protein (ENZO life science, 80-2603) was used to prepare control levels.

cTnI Human Serum Samples (tested with Cobas, Roche) were supplied by Serologix.

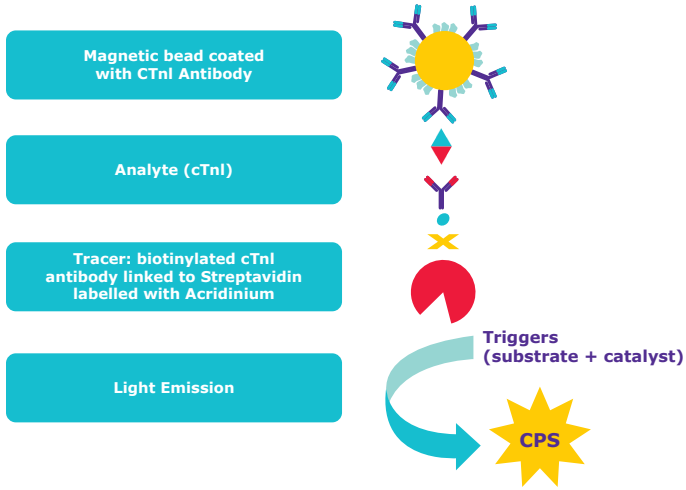
BSA (MERCK, Ref. 810033), was used to block the beads after antibody conjugation and store the beads.

NHS (SIGMA, 56485) and EDAC (SIGMA, E1769) were used to activate the beads during coating procedure.

Assay Scheme

Primary monoclonal cTnI antibody is covalently conjugated to the carboxyl superparamagnetic beads. After incubating the beads with the sample, a secondary monoclonal cTnI biotinylated antibody is added followed by the tracer, which is based on a streptavidin labelled with acridinium. Finally, triggers are added to record the chemiluminescent signal in CPS (counts per second) measured by a commercial chemiluminometer.

Assay Overview



Coating Procedure

A coating procedure was optimised in order to link the capture antibody onto the beads - amount of antibody attached per milligram of beads, incubation conditions, and formulation of buffers involved were modified along the development until a functional coating procedure was achieved.

Carboxyl superparamagnetic beads with PA 8, 15 and 20 were conjugated with the primary monoclonal cTnI antibody under the same parameters and conditions previously optimized.

Covalent conjugation of cTnI monoclonal antibody to superparamagnetic beads consists of the following steps:

1. Beads are washed twice at 1% (w/v) with 50 mM MES pH 6 activation buffer.
2. Carbodiimide and N-hydroxysuccinimide are added 5 times in excess onto the beads at 1% (w/v) to activate the carboxylic acid groups. It is placed under agitation for 15 minutes at room temperature.
3. After activation, a washing step of the beads at 1% (w/v) is performed with 50 mM PBS pH 7.4 coupling buffer.
4. Then beads are resuspended at 2% (w/v) in 50 mM PBS pH 7.4 coupling buffer and 9 µg of primary monoclonal antibody is added per mg of beads getting the beads resuspended at 1% (w/v). It is incubated under agitation for 2 hours at room temperature.
5. Beads are washed twice at 1% (w/v) for 30 minutes with 50 mM Tris, 1% BSA, pH 7.4 blocking buffer under agitation at room temperature. It is then blocked for 1 hour under the same conditions.
6. Beads are washed twice at 1% (w/v) with 50 mM HEPES pH 7.4 storage buffer. Beads are finally resuspended at 1% (w/v) and stored at 2-8 °C.

Tracer

Streptavidin conjugated with acridinium is incubated with the monoclonal cTnI biotinylated antibody for 30 minutes at room temperature under gentle agitation and protected from light. Tracer is stored at 2-8 °C protected from light.

Assay Procedure

Formulation of buffers, immuno reagents volume, incubations, washings steps and reading parameters involved in the assay were modified in order to optimize the assay procedure.

Washes and incubations along the assay were performed manually in a 96-well plate, whereas triggers addition and measurement of luminescence were carried out by a benchtop plate reader.

The assay procedure consists of the following steps:

1. Coated beads are washed twice with 50 mM PBS pH 7.4 with 0.05% (v/v) Tween followed by resuspension at 0.2% (w/v).
2. 150 µl of sample is added to 25 µl of coated superparamagnetic beads at 0.2% (w/v) and incubated for 1 hour on a plate shaker under gentle and continuous orbital agitation at room temperature protected from light.
3. Supernatant is removed and 100 µl of tracer solution is added and incubated for 1 hour under gentle and continuous orbital agitation at room temperature.
4. Four washes with 50 mM PBS pH 7.4 with 0.05% Tween (v/v) are carried out to get rid of the excess label.
5. 50 µl of each trigger is dispensed and chemiluminescent is measured in CPS.

RESULTS

Functional comparison between Carboxyl Magnetic Estapor® Microspheres of 1 µm, with PA = 8, 15 and 20 was assessed for the detection of cTnI by chemiluminescence immunoassay.

Response of the assay evaluated with controls

Spiked controls were used to cover the analytical range response of interest in order to compare the functionality of magnetic beads with different parking areas (PA).

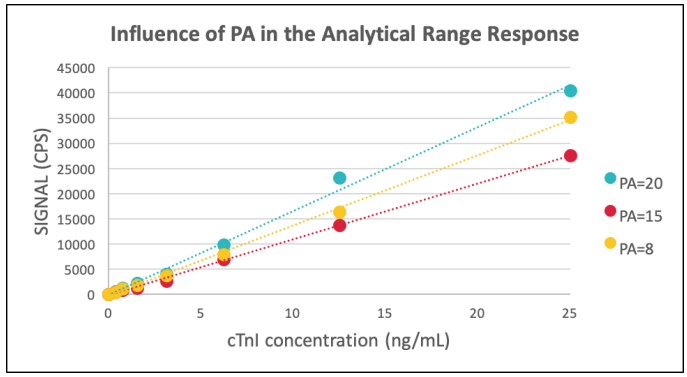


Figure 1. Functionality of the assay up to 25 ng/mL of Troponin-I with magnetic beads with different PA. Each point corresponds to the average of 2 replicates tested with each control.

There was a positive direct relationship between the signal and the cTnI concentration for all PA evaluated. Higher response was displayed by superparamagnetic beads with PA 20 (Fig. 1).

In order to assess the analytical sensitivity for each PA at low concentrations, 7 control levels from 0.05 to 0.50 ng/mL of cTnI were prepared. Six replicates were tested for each level to evaluate the precision at these levels (Fig. 2 and 3).

Beads with PA 20 also showed the best analytical sensitivity at low cTnI concentrations compared to the other beads assessed (Fig. 2). For all cTnI concentrations tested, beads with PA = 20 displayed the highest signal. On the other hand, beads with PA = 15 showed lower response and beads with PA = 8 did not clearly distinguish between the levels tested at the low end concentration range (Fig. 2).

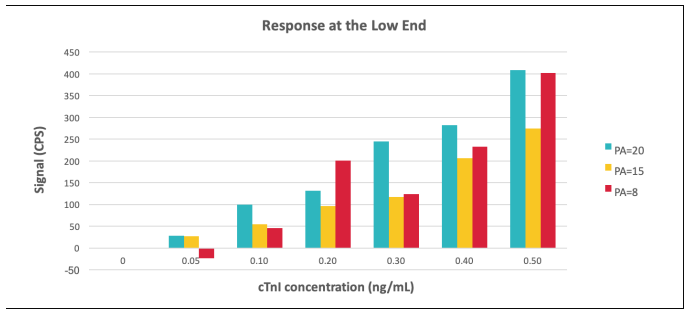


Figure 2. Functionality of the assay at low concentrations up to 0.50 ng/mL of Troponin-I with magnetic beads with different PA. Each bar corresponds to average of 6 replicates tested with each control.

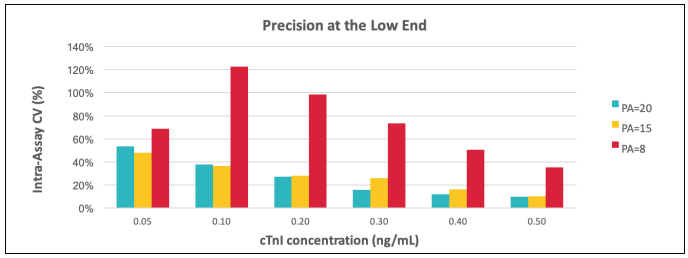


Figure 3. Intra-assay precision assessed at the low end of the analytical range up to 0.50 ng/mL of Troponin-I with controls. Coefficient of variation (%) was obtained with 6 replicates tested at each concentration.

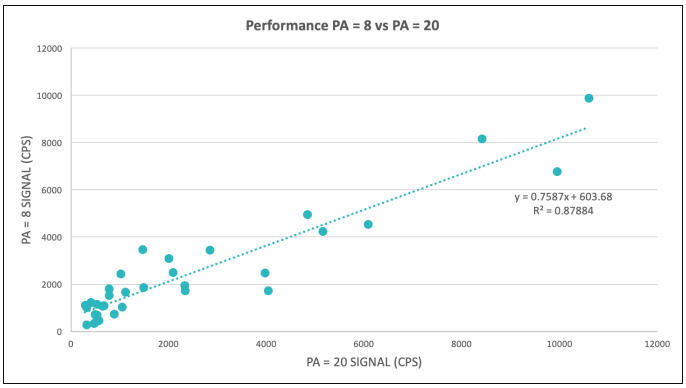
The lower the analyte content in the sample, the lower the signal is displayed so that the coefficient of variation usually gets higher. Results shown in Figure 3 displayed high CV (%) values at low concentrations, indicating that the assay requires further optimization. However, the study showed valuable information to determine the best parking area to continue with the development. Superparamagnetic beads with PA 20 showed the lowest CV values, which were below 20% from 0.30 ng/mL to 0.50 ng/mL of cTnI (Fig. 3). Beads with PA 8 showed very high CV (%) and beads with PA 15 displayed a similar precision compared to beads with PA 20.

Performance of the assay evaluated with serum samples

Human serum samples were collected, tested by Roche test in a Cobas platform and stored at -80°C by the supplier.

Once received, samples were stored at -80°C and defrosted just prior to performing the testing. Samples were thawed at room temperature and tested within the next 3 hours.

33 Human serum samples that cover the analytical range response of interest were tested in duplicate under the same parameters and conditions to compare the performance of beads with PA 8, 15 and 20 (Fig. 4a and 4b).



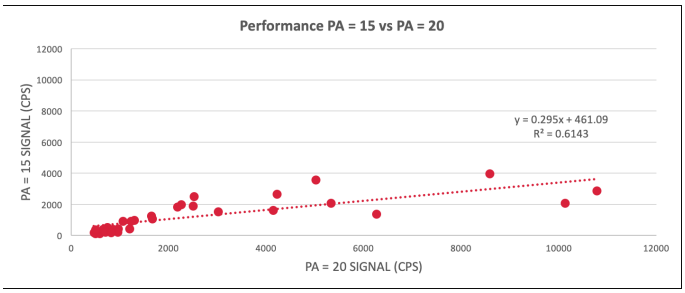


Figure 4 (a and b). Performance of beads are confronted by pairs. Average signal (CPS) of two replicates of each human serum sample tested are displayed in the graphs.

The three lots of beads showed a trend of signal that increases with the cTnI concentration in human serum samples.

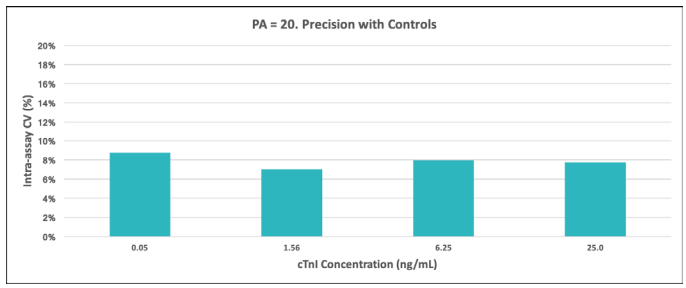
Beads with PA 20 and 8 displayed higher slope and similar performance. However, PA=8 did not clearly distinguish between the samples with low concentration as it was expected according to Figure 2 where controls were assessed (Fig. 4a).

On the other hand, beads with PA 15 showed low analytical sensitivity (Fig. 4b), probably due to low response as it was shown in Fig. 1.

Precision of assay with PA = 20

Based on the previous results shown (performance of the assay with controls and samples), superparamagnetic beads with PA 20 were selected to carry out with further studies.

The precision of the assay with superparamagnetic beads PA = 20 was determined by calculating the intra-assay CV (%) of 4 levels of cTnI controls and 4 levels of cTnI serum samples that cover the analytical range of interest. 10 replicates of each level were assessed in the same run.



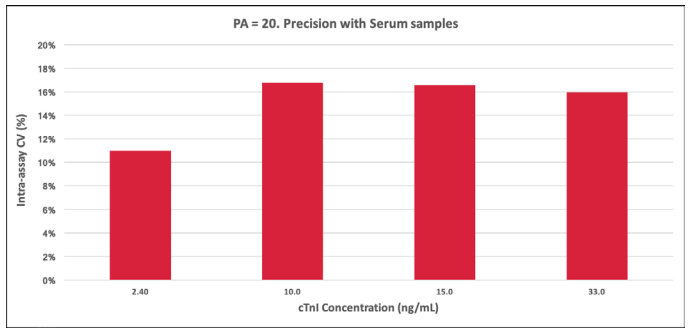


Figure 5 (a and b). Intra-assay precision with beads PA=20. Coefficient of variance (%) is calculated with 10 replicates tested for each level.

Intra-assay precision assessed with controls was below 10% (Fig. 5a).

Higher CV (%) values were displayed when serum samples were tested. Serum matrix seems to have an effect on the precision of the assay (Fig. 5b).

Stability of the assay with beads PA = 20

Stability of superparamagnetic beads with PA 20 was assessed by storing conjugated beads at 4 °C and 37 °C for 14 days.

Stability of beads stored at 4 °C represents the real time stability, which can be used to monitor their shelf life, whereas beads stored at 37 °C displays the

Table 1

	0.00 ng/mL cTnI	1.56 ng/mL cTnI		6.25 ng/mL cTnI		25.0 ng/mL cTnI	
	Signal (CPS)	Signal (CPS)	% Related to 4 °C	Signal (CPS)	% Related to 4 °C	Signal (CPS)	% Related to 4 °C
4 °C (day 14)	0	1194	100%	6596	100%	23814	100%
37 °C (day 14)	16	1148	96%	6994	106%	26228	110%

Table 1. Signal displayed by beads stored at 4°C (real time stability) vs beads stored at 37°C for 14 days (accelerated stability). Differences of signal related to beads stored at 4°C are highlighted in blue (%).

accelerated stability study. The accelerated stability simulates aging of the beads, thereby predicting instability of the reagent in advance.

After 14 days, beads stored at 4 °C and 37 °C were evaluated under the same parameters and conditions in the same run by testing 4 levels of cTnI controls in triplicate.

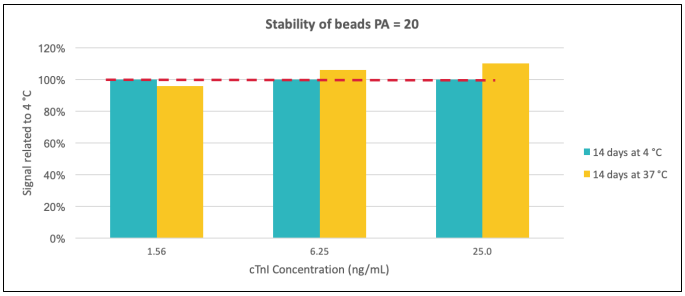


Figure 6. Comparison of signal displayed (in percentage %) related to beads stored at 4 °C.

The biggest difference of signal with beads stored at 37 °C goes up to 110% (Fig. 6), which is within the Coefficient of Variation observed in the precision study (Fig. 5a).

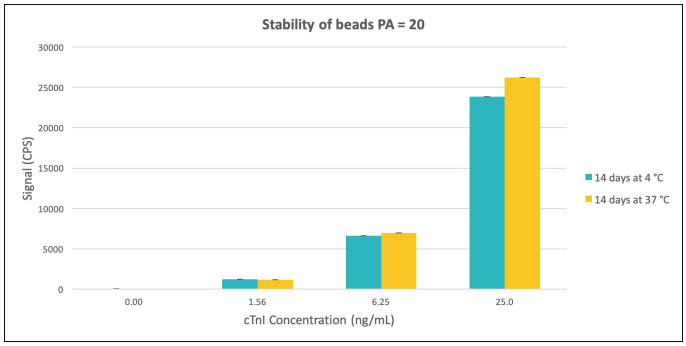


Figure 7. Signal displayed with beads stored at 4 °C vs 37 °C. Each bar corresponds to the average of three replicates tested for each Troponin-I control.

Similar response has been observed after having stored the beads at 37 °C for 14 days (Fig. 7).

CONCLUSION

According to this study, beads with PA = 20 displayed the highest response and highest analytical sensitivity when tested under the same conditions. They also showed the best precision at low cTnI concentrations, what gives confidence to point out that the beads with PA = 20 displayed the best performance.

The fact that results from accelerated stability studies (14 days at 37 °C) are very similar to those obtained with beads stored at 4 °C demonstrates the robust stability of the conjugated beads.

Particles with the lowest parking area (PA = 8) displayed the worst precision. Among the evaluated particles, these are the ones with more content of carboxyl groups. A possible hypothesis could be that epitope antibodies can be less accessible when content of carboxylic groups is higher on the beads surface. This could have an effect in the precision and analytical sensitivity at the low end concentration of the assay due to steric effects.

According to the results shown in this technote, the beads with PA = 20 seem to be more suitable for this kind of assay.

Nevertheless, we highlight the critical importance of evaluating superparamagnetic beads with different content of functional groups during the development of a CLIA immunoassay in order to make sure the best paramagnetic beads are selected.

REFERENCES/BIBIOGRAPHY

1. Soetkamp D, Raedschelders K, Mastali M, Sobhani K, Bairey-Merz C.N, Van Eyk J. The continuing evolution of cardiac troponin I biomarker analysis: from protein to proteoform. Expert Rev Proteomics. 2017 December; 14(11):973-986. Doi: 10.1080).
2. Jaffe AS, Vasile VC, Milone M, Saenger AK, Olson KN, Apple FS. Diseased skeletal muscle: a noncardiac source of increased circulating concentrations of cardiac troponin. Journal of the American College of Cardiology. 2011 Oct 18;58 (17):1819-24. Doi:10.1016).

To place an order or receive technical assistance

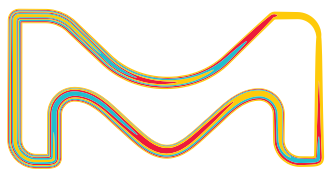
In Europe, please call Customer Service:

France: 0825 045 645	Spain: 901 516 645 Option 1
Germany: 069 86798021	Switzerland: 0848 645 645
Italy: 848 845 645	United Kingdom: 0870 900 4645

For other countries across Europe, please call: +44 (0) 115 943 0840
Or visit: **MerckMillipore.com/offices**
For Technical Service visit: **MerckMillipore.com/techservice**

MerckMillipore.com

Merck KGaA
Frankfurter Strasse 250
64293 Darmstadt, Germany



Lit. No. MK_TN4644EN Ver. 0.0
2019 - 24939
08/2019