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SANTA CRUZ

A SURROGATE BASED CHARACTERIZATION TECHNIQUE

FOR ANTIBODY COUPLING EFFICIENCY

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of the requirements for the degree of

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in

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by

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Abstract

A Surrogate Based Characterization Technique

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The immunoprecipitation protocol is of key importance to antigen purification, to downstream protein analysis, and to clinical research. The success of an immunoprecipitation experiment depends on many variables of which antibody immobilization is the most important. Antibodies must be coupled with specific solid surfaces, such as agarose beads or superparamagnetic beads, during immunoprecipitation. Although antibody coupling is crucial for the protocol, there is not an easy-to-apply, low cost, or fast method to maximize and characterize the immuno-binding capacity. In this work we propose a characterization method which gives a clear result of the coupling efficiency, which does not require complicated experimental steps or expensive laboratory equipment. By forming a two-particle complex that sandwiches an antibody, the total amount of antibody coupled to the given number of magnetic beads can be readily translated into the number of surrogate beads that are attached to the magnetic beads through the antibodies. This method doesn't require additional biological reagents. The surrogate bead alone suffices to quantify immobilization efficiency. This method requires only fluorescence imaging, which can be done with any portable fluorescence platform. It has multiplexing capability, provides high-throughput and high-yield, is cost-effective

and time-efficient. Researchers can easily track the shelf-life, quality, degradation, and deterioration of the immobilized beads. This research has characterized a couple of variables, as well as tested automating the protocol. Our design also allows other researchers to explore more variables and automate their protocols.

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Chapter 1: Introduction and Background

1.1 Motivation

Recent developments in diagnostic technology for common diseases such as cancer, HIV, and Ebola caused an increasing need for more interdisciplinary research in many areas including engineering, biology, physics, chemistry, material science, and computer science. Because of this, researchers have an opportunity to take their expertise from one discipline and apply it to another research area to investigate missing points.

Diagnostic technology depends on the presence of protein in the medium. Protein purification and separation must be done during disease diagnostics. Immunoprecipitation (IP) is one of the most commonly used protocols for protein antigen purification and separation [1]. Immunoprecipitation is a technique where a primary antibody binds to a specific protein antigen and the combined assay precipitates at the end of the experiment.

Antibodies have to be immobilized on a solid surface to complete the precipitation protocol. This process is called antibody coupling or immobilization. Although antibody immobilization is a commonly used process with well-documented procedures and efficiency graphs, as shown in Figure 1.1, there is no such simple way to verify the efficiency of the protocols and the immuno-binding coupling of antibodies. To overcome this limitation, we developed a new technique enabling us to quantify the efficiency of immuno-binding processes.

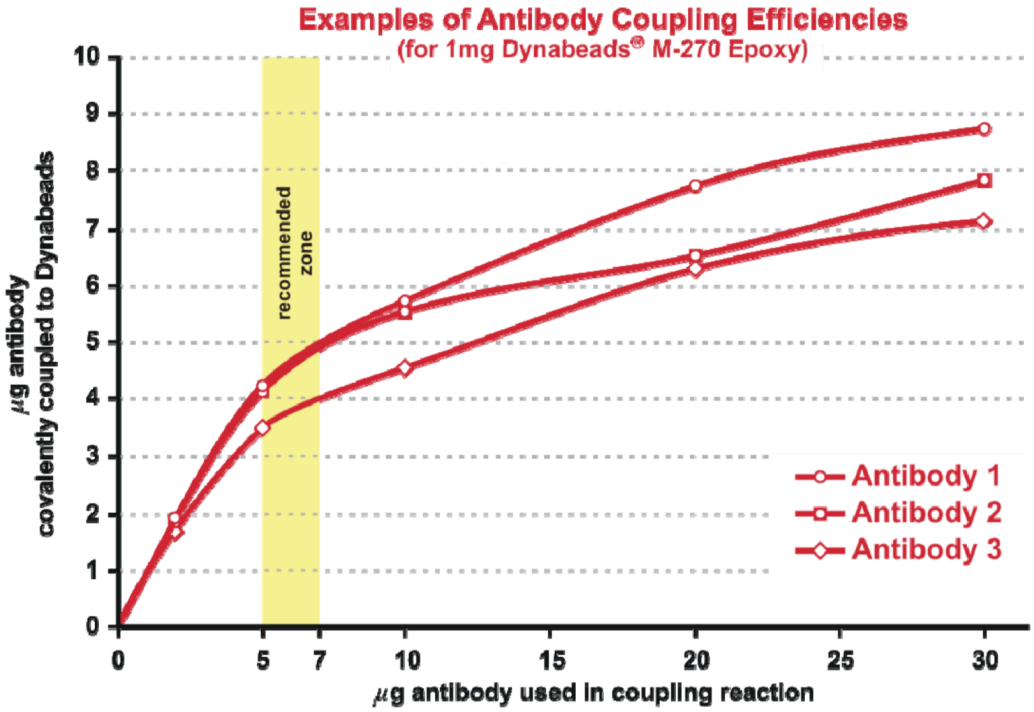

Figure 1.1 – Example of an efficiency graph in an immunoprecipitation reaction procedure. Although the effect on antibody weighs used in coupling reaction shown in this graph, there is no such a method given that lets researchers observe results by themselves. Adapted from [2].

1.2 Immunoprecipitation objective

Immunoprecipitation is a technique in which protein antigen is captured by a specific antibody attached on a solid surface. This solid surface can be either sponge-like non-magnetic agarose beads or superparamagnetic epoxy beads. Although agarose beads used to be the preferred solid surface to immobilize antibodies in the past, there is an increased use of magnetic beads recently, as shown in Figure 1.2.

Immunoprecipitation can be used for many purposes. Protein antigen presence test, cell culturing, quantification of protein synthesis rate, and downstream protein analysis are among these purposes [3].

There must be a step where the antibody is binding on a solid surface during immunoprecipitation. This immobilization step is called as antibody coupling. Antibody coupling could be done before adding specific target proteins, or after antibodies and antigens get attached to each other. The first option is known as pre-immobilized antibody approach, and the latter one called free antibody approach.

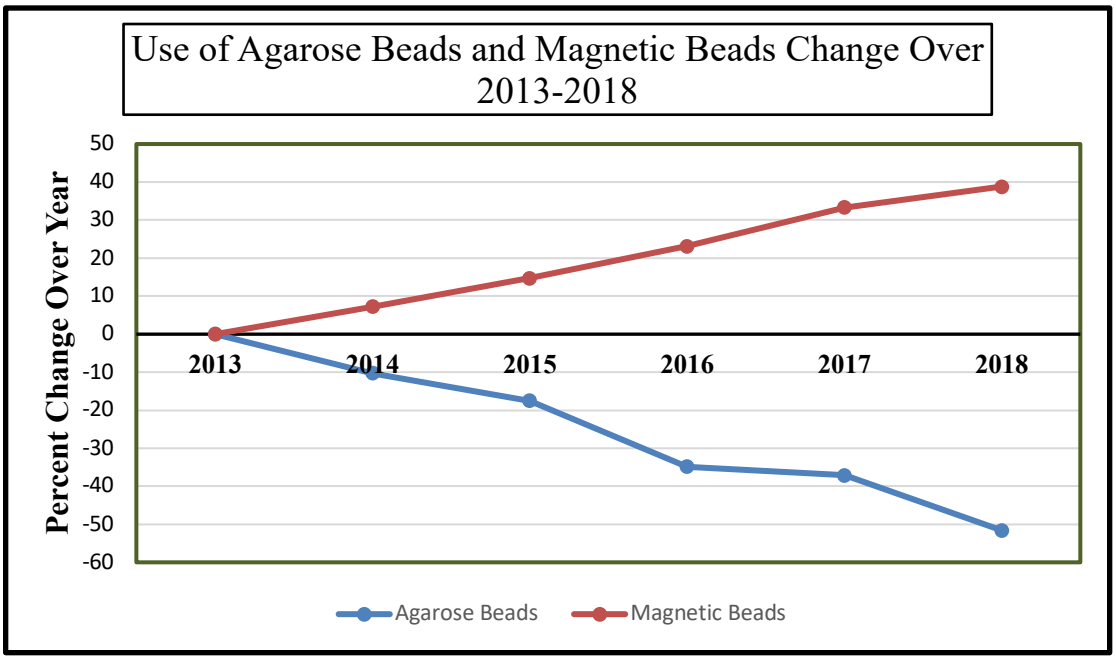

Figure 1.2 - Comparison of the number of papers using different solid supports between 2013 and 2018.

1.3 Antibody Immobilization Methods

Attaching the primary antibody on a solid surface during the immunoprecipitation is called antibody immobilization. The two general immobilization methods for immunoprecipitation are the pre-immobilized antibody approach and the free antibody approach. In this work, we have focused on pre-immobilized antibody coupling efficiency characterization.

1.3.1 Pre-Immobilized Antibody Approach

In pre-immobilized antibody approach, specific antibodies for the target protein are attached on a solid support. This support can be either non-magnetic agarose beads or superparamagnetic beads. After that, the target protein mixture is then added into the antibodies that are already bounded on solid support. Proteins become immunoprecipitated after incubation. The detailed illustration is shown in Figure 1.3.

1.3.2 Free Antibody Approach

In the free antibody immobilization approach, capture antibodies that are specific for a target protein, are added directly to the mixture of protein. These antibodies are not immobilized on a solid surface yet. The antibodies freely floating in the mixture attach to their target proteins during incubation. Solid support is added to the mixture after this first incubation. Antibodies attached to antigens are subsequently captured on these solid supports during incubation [4].

Immunoprecipitation protocol that uses either one of the mentioned immobilization methods converge after antibody-antigen couple are captured on the solid supports.

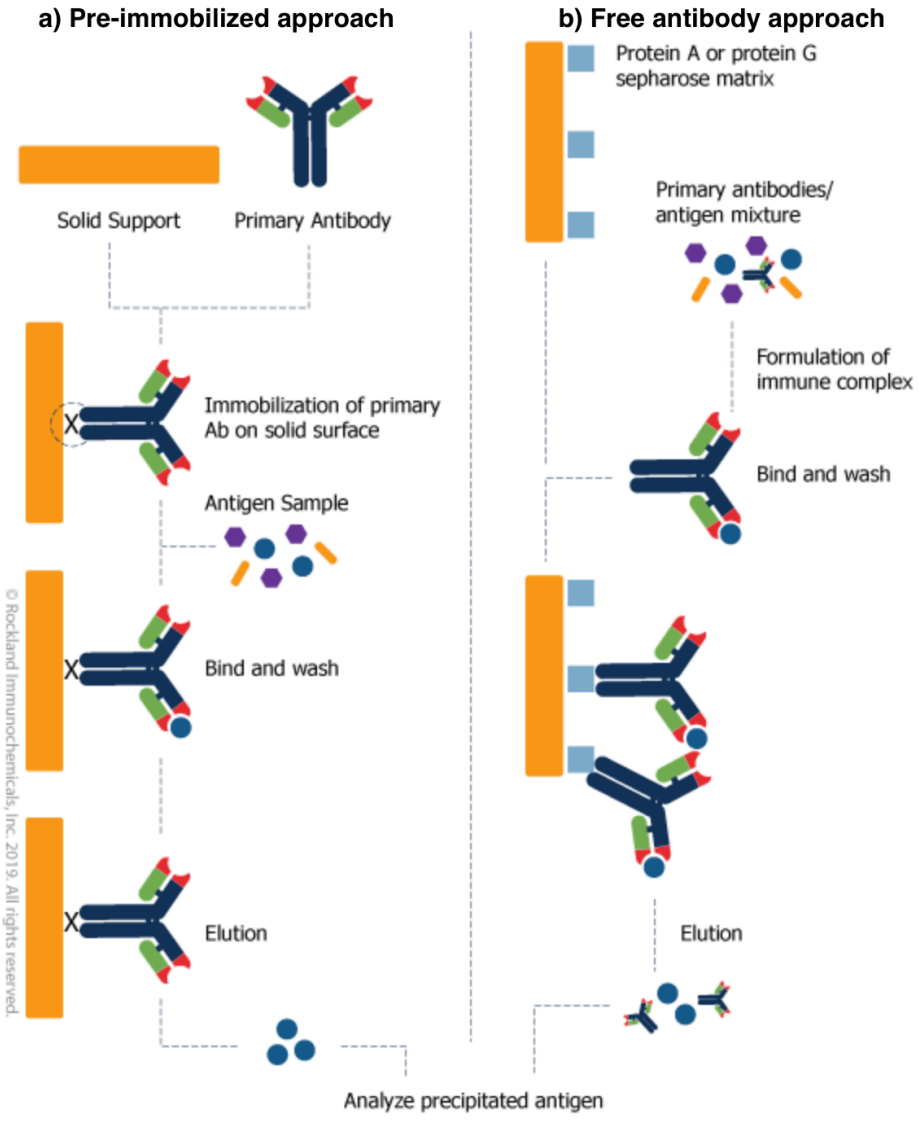

Figure 1.3 – a) Immunoprecipitation protocol with the pre-immobilized antibody approach. b) Immunoprecipitation protocol with the free antibody approach. Adapted from [4].

1.4 Solid Surface Support Types in Immunoprecipitation

There are two types of solid beads frequently used for antibody immobilization. The first type is called agarose beads (non-magnetic), and the other is called superparamagnetic beads. Agarose beads used to be preferred one in the past, as mentioned in the immunoprecipitation objective. However, the number of immunoprecipitation protocols done by using superparamagnetic beads are continually increasing, and we have focused on characterizing magnetic bead immobilization efficiency in this work.

1.4.1 Agarose Beads

Sponge-like shaped, highly-porous agarose beads have been the most frequent researchers' choice for solid support for immunoprecipitation protocol. Agarose bead size fluctuates between around 50 micrometers to 150 micrometers [5]. They have a very high potential binding capacity because of many cavities present in their sponge-like structure, which are available for antibody bonding. Immunoprecipitation protocol can be performed without the need for any specialized equipment when agarose beads are the solid support. An SEM image of the agarose beads is shown in Figure 1.4.

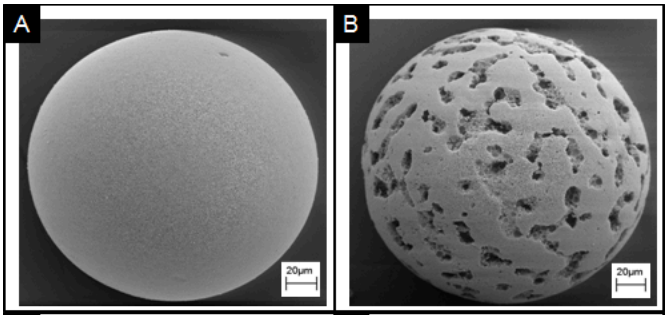

Figure 1.4 – SEM images of a) Surface morphology of the 4% agarose containing beads. b) Superporous beads containing 4% agarose with ~30 µm microcavities. These microcavities increases the capacity of antibody immobilization. Adapted from [6].

When antibody bonding saturation is not necessary for the IP protocol, the cavities that provide high binding capacity can be disadvantageous. Usually, it happens when the amount of antibody attached on the agarose bead is less than the sufficient amount for binding saturation. In these cases, agarose beads will be coated partially with the antibodies, while the non-coated surface is open to bind anything else in the medium. This binding is also called non-specific binding, causing a larger noise level. The background signal can be reduced by pre-clearing the lysate, which is recommended for any immunoprecipitation protocol in general [7], [8].

1.4.2 Superparamagnetic beads

Although the majority of immunoprecipitations were performed with non-magnetic agarose beads in the past, the popularity of superparamagnetic beads, which is a newer method for immunoprecipitation protocol, is continuously increased over

the last decade as shown in Figure 1.2. Due to the solid and spherical shape of the magnetic beads, antibody binding is limited to the surface of every single bead.

Magnetic beads are significantly smaller than agarose beads (few μm vs. >50 μm) and do not have a porous shape that allows as many antibodies to bind on its surface. However, the increased of number of the magnetic beads provides better surface-area-to-volume ratio for immobilization, and overcomes its shape disadvantage against agarose beads. A sample SEM image of the Dynabeads M-270 Epoxy bead is shown in Figure 1.5.

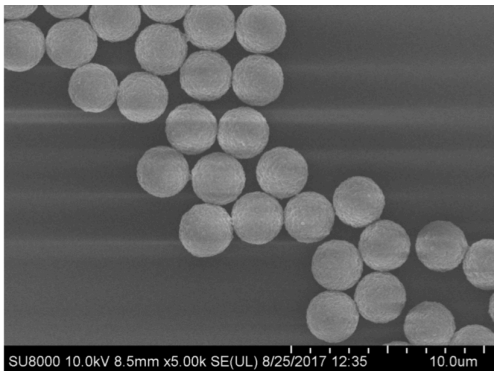

Figure 1.5 – An SEM images of a Dynabeads M-270 epoxy based superparamagnetic beads with a diameter of 2.8 μm. Adapted from [9].

There are two types of beads available in the commercial market in monodisperse and polydisperse form. Their difference is their size uniformity. Monodisperse beads display exact uniform size, which provides identical binding characteristics, such as capacity and level of attraction to magnets. Polydisperse bead's size is also similar to monodisperse beads. However, their size is not uniform and varies between 1 micrometer to 4 micrometers [10], [11]. This fluctuation affects

the binding characteristics as mentioned for monodisperse beads. High-quality monodisperse beads are ideal for immunoprecipitation protocol because of their physical size, shape consistency, and level of attraction to the magnets. Monodisperse beads also allow automation of the protocol.

Increased preference of magnetic beads compared to the agarose beads relies on the claim that magnetic beads have a faster protein binding than agarose beads for IP applications [12]–[14]. Researchers claimed that magnetic beads are more efficient for extremely large protein immunoprecipitation because of the missing size limits for these complexes [12], [13], [15].

Moreover, sample separation in magnetic bead technology does not require any centrifuge, which means that physical stress reduces and sample handling is more manageable [13]. Agarose bead-based sample separation involves harsh centrifuge steps and results in fragile protein to antibody attachments [13]–[15].

Additional factors such as binding capacity, yield, reproducibility, purity, speed, automation, etc. will be discussed in detail in the next part of this chapter.

1.5 Agarose Beads versus Superparamagnetic Beads

1.5.1 Binding Capacity and Yield

When comparing agarose beads and magnetic beads, agarose beads seem to have the advantage of having much greater surface area due to its sponge-like structure. But that does not mean that this advantage will result in a higher yield. Although more antibodies attached to the agarose beads during the immobilization, it is more likely that antigens cannot find their way to the antibodies during incubation

because most of the antibodies are attached to the inside of the sponge-like shape cavities. Also, the sponge-like shape will result in the loss of antigen proteins during harsh wash steps that are performed after centrifuging the assay.

On the other hand, antibodies that are immobilized on uniform magnetic beads surface is more stable and will not be washed away during the gentle washing steps. Washing steps for magnetic bead-based assay are performed with a magnet that collects the assay onto the inner wall of the centrifuge tube. Hence, even if the antibody-binding capacity is lower for magnetic beads, the final protein yield is similar, or often higher, than agarose beads.

1.5.2 Reproducibility and Purity

Non-specific binding is always a problem that researchers encounter regularly. When the antibody binds to unintended antigen proteins, we can speak about non-specificity which causes background signal and noise. Non-specific binding is demonstrated in Figure 1.6.

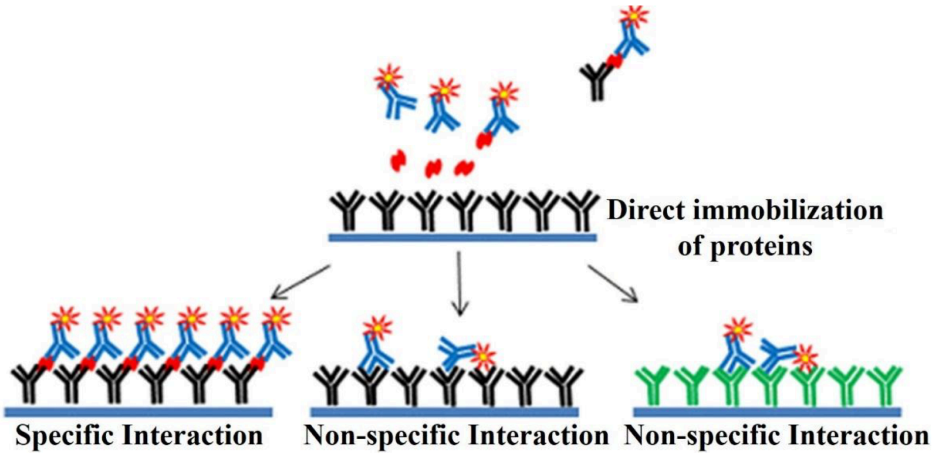

Figure 1.6 – Non-specific protein binding demonstration. Adapted from [16].

Removing the buffer without touching the pelleted resin in reactions that use agarose beads is not an easy task. On the other hand, when the magnetic-bead based IP reactions are performed, all the precise bead complexes are firmly held on the magnet side of the centrifuge tube. This allows removing the wash buffer without touching the bead-pellet. Resuspending the bead-pellet is possible when the magnetic field is removed since the magnetic beads immediately lose their magnetic remanence. A detailed demonstration of removing the wash buffer shown in Figure 1.7.

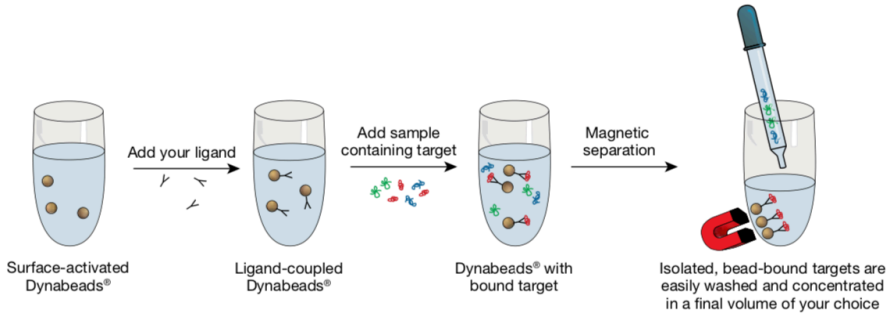

Figure 1.7 – Example of a commercial product, superparamagnetic Dynabeads for reproducibility and purity. Magnetic beads allow for flexible application. They provide gentle washing steps against specific targets. Magnet pellets the beads that are antibodies and captured target proteins on them, and experimentalist can easily remove the wash buffer without harming the assay. Adapted from [17].

In addition to this, agarose bead-based reactions require more incubation time because of its sponge-like shape and pre-clearing the lysate to reduce non-specific binding. On the other hand, the smooth and uniform surface of the magnetic beads requires less amount of incubation time, and doesn't require any pre-clearing. These

advantages provide higher reproducibility and purity in magnetic bead-based IP reactions.

1.5.3 Speed, Ease of Use and Automation

The aforementioned advantages of magnetic beads versus agarose beads explain the increased preference for magnetic bead-based IP reactions. Magnetic bead-based reactions usually require incubation times around half an hour to complete, while agarose bead-based reactions require between one hour to one and a half hours. The magnet-stands often come in an array format which allows doing reactions higher throughput. The amount of data obtained with magnetic bead-based reactions is significantly increased compared to its alternative, agarose beads.

In addition, repetitive experiments for IP based reactions can be easily automated using magnetic beads. This option will be beneficial for researchers to reduce the amount of work and save time. Researchers will also obtain more accurate results. They can perform high-throughput applications with using 96-well plates [18].

1.5.4 Equipment

Other than standard laboratory equipment for immunoprecipitation reactions, magnetic bead-based IP reactions do need an additional magnetic field. To provide this field, an easy-to-use simple magnet stand with a centrifuge-tube holder rack will

be sufficient. Comparing the agarose bead-based protocol, which requires 24-hours at 4 °C, to magnetic bead-based reaction which can be completed in one hour would provide more data in a shorter time [12], [14]. Considering this big difference in time, an additional magnet stand will be a feasible expenditure to increase the obtained data.

1.5.5 Summary

Agarose beads have more binding capacity than magnetic beads. However, considering the yield, reproducibility, purity, ease of use and more importantly automation, using magnetic beads for the immunoprecipitation reaction is a better option. Performance comparison of the agarose bead-based and the magnetic bead-based IP protocol is shown in Table 1.1.

	Capacity	Yield	Reproducibility	Purity-Specificity	Ease of Use	Speed	Automation
Agarose Beads	***	**	**	*	*	*	N/A
Magnetic Beads	**	***	***	***	****	****	***

Table 1-1- Performance comparison of the agarose beads and magnetic beads as solid surface support in immunoprecipitation reaction. Adapted from [19].

Chapter 2: Methods

This section discusses the methods for antibody immobilization, reagent preparation, surrogate bead bonding procedure, fluorescence imaging, and the automation of the whole procedure. All the experiments were done at the Nanoengineering Group facilities at the University of California Santa Cruz under the supervision of Prof. A. Ali Yanik. Thermo Fisher Scientific Dynabeads® Antibody Coupling Kit (Catalog number: 14311D) was selected as the solid surface support for antibody immobilization. Fluorescence imaging was performed using a Nikon Eclipse TiE series fluorescence microscope. Data analysis and plotting graphs were performed with MATLAB.

2.1 Antibody Immobilization

2.1.1 Immobilization Considerations

The selection of appropriate antibody is the most critical factor for successful target protein capture [2]. Not all the antibodies bind target proteins. Antibody and target protein pair match must be determined and confirmed before starting antibody immobilization. Also, it should be considered that while some pairs work for methods such as western blotting, it does not necessarily mean that the same pair would work for immunoprecipitation protocol too.

Low quantities of ligands should be used per milligram of magnetic beads. Optimal immobilization occurs at 5-10 micrograms of antibody per milligram of magnetic beads. The sample curve was shown in Figure 1.1. However, if the cost is

not a restrictive factor, the researcher can go up to 20-30 micrograms of the antibody per milligram of the magnetic beads. Purified ligand increases the coupling efficiency. Saturating the amount of antibody will also help to reduce the background noise [2]. An excess of the saturation might result in antibody leakage. Increasing the number of washing steps will help to minimize antibody leakage.

In addition, the buffer solution for antibody or antigen should not be glycerol-based since glycerol-based buffers slow down the bonding process that results with increase time for the incubation.

2.1.2 Immobilization Protocol

In this work, the immobilization protocol was followed that was provided by Thermo Fisher Scientific with Dynabeads® Antibody Coupling Kit. The amount of the solution used may change upon weighing of the M-270 epoxy beads.

A magnetic stand is required. We have used the DynaMag™-2 magnet stand (Catalog number: 12321D). This stand comes with a vial rack and can hold 16 microcentrifuge tubes at a time. A rotator/mixer is needed to perform the mixing of the solutions. Thermo Fisher Scientific HulaMixer™ Sample Mixer (Catalog number: 15920D) was selected for this purpose. This rotator allows processing 26 microcentrifuge tubes simultaneously, which enables us to test more than one variable in a single experiment. Pictures of both the magnet stand and the rotator are shown in Figure 2.1.

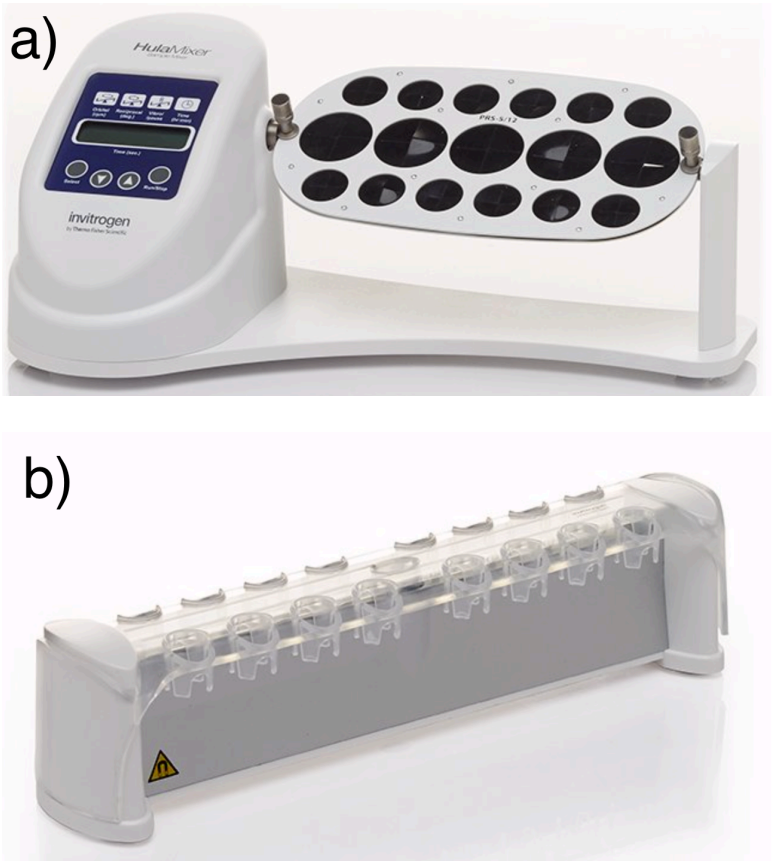

Figure 2.1 – a) HulaMixer™ Sample Mixer. b) DynaMag™-2. Adapted from [20], [21].

Biotin-conjugated HIV antibodies are one of the commonly available ones in the market. We have used Mybiosource Inc. mouse immunodeficiency virus monoclonal antibody (Catalog number: MBS568008). This antibody is compatible with epoxy based magnetic beads and surrogate beads.

A wash buffer selection is also vital for immobilization efficiency. Phosphate Buffered Saline was the preferred wash buffer in our experiments due to its proven compatibility with immunoprecipitation protocol over the years. We have used

Gibco™ 1X PBS with 7.4 pH (Catalog number: 10-010-023). Thermo Scientific™ Blocker™ BSA (10% BSA) was used for blocking purposes (Catalog number: PI37525).

One milligram of M-270 magnetic beads were used for each experiment to minimize cost. Typically, this amount is enough for immunoprecipitation protocols. For the antibody immobilization, the following procedure has been performed. This protocol is quoted from [2].

Note: Some steps are modified and optimized for our purpose.

Note: C1, C2, HB, LB, and SB are the wash buffers provided with this kit that are used for different steps of the immobilization.

1. Disinfect the magnet you will be using to prevent accidental sample contamination.

2. Weigh out the appropriate amount of Dynabeads® M-270 Epoxy. Table for calculation antibody and magnetic bead volumes is shown in Table 2-1.

3. Wash the beads with 1 mL of C1 and mix by vortexing or pipetting.

4. Place the tube on a magnet for 2 minutes and allow the beads to collect at the tube wall. Remove the supernatant.

5. Add the appropriate volume of antibody + C1 (see Calculation of Antibody and C1 Volumes in the following table) to the washed beads and mix by gentle vortexing or pipetting. Example: If you are coupling 5 mg Dynabeads® and your

required quantity of antibody has a volume of 100 µL, you need to add 150 µL of C1 (i.e., 250 µL C1 – 100 µL Ab = 150 µL.)

6. Add the appropriate volume of C2 and mix by gentle vortexing or pipetting.

7. Incubate on a roller at 37°C overnight (16–24 hours). If the incubation is performed at room temperature, incubation time could be increased a few hours.

Overnight incubation is important for the success of the immobilization. The researcher should make sure that the mixture in the vial is mixing well and beads do not settle while rotating. It is also important to make sure that the mixture is not flowing inside the vial while the vial is turned upside-down. That phenomena occurs when a small amount of volume is dispensed into the vial. 100 microliters of the mixture should stand together because of its surface tension against the vial.

	Volume (microliters)		
Beads (mg)	**C1**	**C2**	**Total Volume**
1	50 - Vol Ab.	50	100
5	250 - Vol Ab.	250	500
20	1000 - Vol Ab.	1000	2000

Table 2-1- Calculation of the C1, C2, and the antibody volumes. C1+antibody volume should be equal to C2 volume. The total volume should be 100 microliters per each milligram of the magnetic beads. Adapted from [2].

8. Place the tube on a magnet for 2 minutes and allow the beads to collect at the tube wall. Remove the supernatant.

9. HB wash: Add the appropriate volume of HB and mix by vortexing or pipetting. The appropriate amount of calculation is shown in Table 2-2.

	Volume (microliters)		
Beads (mg)	**HB**	**LB**	**SB**
<20	800	800	800
≥20	1600	1600	1600

Table 2-2- Calculation of HB, LB and SB wash buffer. Adapted from [2].

10. Place the tube on a magnet for 2 minutes and allow the beads to collect at the tube wall. Remove the supernatant.

11. LB wash: Add the appropriate volume of LB and mix by vortexing or pipetting. The appropriate amount of calculation is shown in Table 2-2.

12. Place the tube on a magnet for 2 minutes and allow the beads to collect at the tube wall. Remove the supernatant.

13. Short SB wash: Add the appropriate volume of SB and mix by vortexing or pipetting. The appropriate amount of calculation is shown in Table 2-2.

14. Place the tube on a magnet for 2 minutes and allow the beads to collect at the tube wall. Remove the supernatant.

15. Repeat Short SB wash once more. (Note: If antibody leakage is determined to be a problem repeat this step one or two more times.)

16. Long SB Wash: Add the appropriate volume of SB and mix by vortexing or pipetting.

17. Incubate on a roller/rotator at room temperature for 15 minutes.

18. Place the tube on a magnet for 2 minutes and allow the beads to collect at the tube wall. Remove the supernatant.

19. Resuspend antibody-coupled beads in 100 µL 1% BSA per mg beads and store at 2°C to 8°C until use. The final bead concentration is 10 mg/mL antibody coupled beads.

20. If desired, antibody-coupled beads may be concentrated up to 30 mg/mL by reducing the storage buffer volume. Beads are now covalently coupled with the antibody and ready for immunoprecipitation protocol.

2.2 Reagent Preparation for the Surrogate Bead Bonding

The reagents for the surrogate bead conversion protocol are prepared by the time antibody immobilization is finalized. Wash buffer, elution buffer, surrogate beads, and experiment vials are needed for our protocol.

After testing a few different concentrations, 1% BSA buffer has chosen for wash buffer. Thermo Scientific™ Blocker™ BSA (10% BSA) was used (Catalog number: PI37525) was diluted 1:10 ratio in the Gibco™ 1X PBS with 7.4 pH (Catalog number: 10-010-023) to obtain wash buffer.

Molecular Probes™ FluoSpheres™ NeutrAvidin™-Labeled Microspheres (Catalog number: F8774) was used as surrogate beads. These beads are dielectric and shows yellow-green fluorescent behavior at 505/515 nanometers wavelength. These surrogates have a diameter around ~0.17 μm and Scanning Electron Microscope (SEM) image of the surrogates is shown in Figure 2.2. The number of beads in the stock vial is 1e12/ml. Stock concentration serially diluted to obtain 1e9/ml concentration for binding purposes. 1% BSA buffer used for the dilution.

Thermo Scientific™ Pierce™ Binding and Elution Buffer (Catalog number: PI21004) was used as the elution buffer after the surrogate bead conversion is completed.

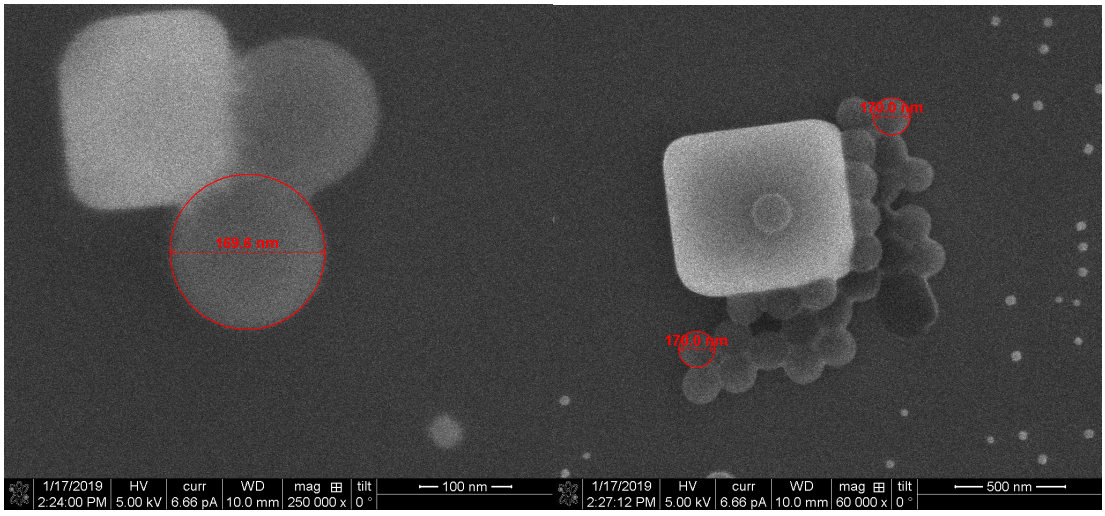

Figure 2.2 – a) A sample image of the surrogate beads. b) Surrogate bead cluster. Images are taken by using Dual-Beam Microscope (Quanta 3D FEG) at the W. M. Keck Center for Nanoscale Optofluidics at the UCSC.

Microcentrifuge tubes were obtained from Fisherbrand™ Snap-Cap™ (Catalog number: 02-681-271). The antibody and the target surrogate beads leak out during the wash process when the microcentrifuge tubes are not blocked. These tubes were blocked for 1 hour beforehand with a 5% BSA solution to decrease the non-specific binding and sample loss with leakage. HulaMixer™ Sample Mixer was used during the blocking.

2.3 Biomarker-to-Surrogate Bead Conversion

Characterization of the efficiency of antibody immobilization has three main parts. The first one was the antibody immobilization, which we discussed in sections 2.1 and 2.2. The second and critical part is biomarker-to-surrogate bead conversation. We have created a two-particle complex sandwich assay to convert biomarkers into the surrogate beads, which can be detected using their fluorescence signal. Our biomarkers are the antibodies which were immobilized onto the magnetic beads in advance. Surrogate beads are bonded to this assay, and antibodies are sandwiched between the magnetic bead and surrogate bead. Sample demonstration of the design is shown in Figure 2.3

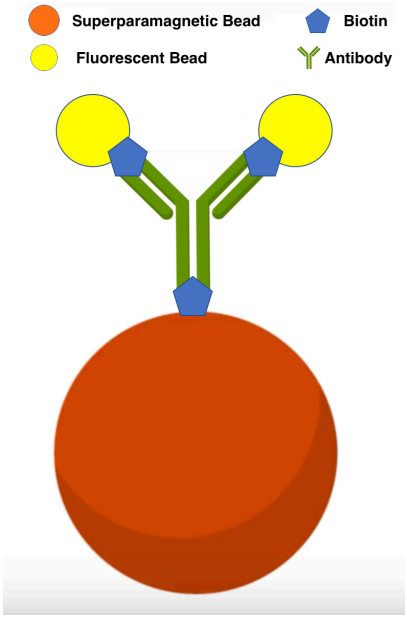

Figure 2.3 – Two-particle complex sandwich assay. First, the biotinylated antibodies are immobilized on the magnetic beads. After that, the surrogate beads added into the medium to create a sandwich assay.

Performing this conversion is very similar to the antibody immobilization steps. The immobilized beads, wash buffer, and other reagents should be prepared to perform surrogate bead conversion. Conversion steps are as follows. These steps are demonstrated in Figure 2.3, 2.4, and 2.5.

1. Take a blocked centrifuge tube out and empty its blocking buffer.

2. Weigh out the appropriate amount of immobilized beads and dispense it into the empty blocked centrifuge tube.

In our case, we will use ten microliters of the immobilized beads, which is one-tenth of the product prepared in Section 2.1.

3. Place the tube on a magnet for 2 minutes and allow the beads to collect at the tube wall. Remove the supernatant. Remove the tube from the magnet stand.

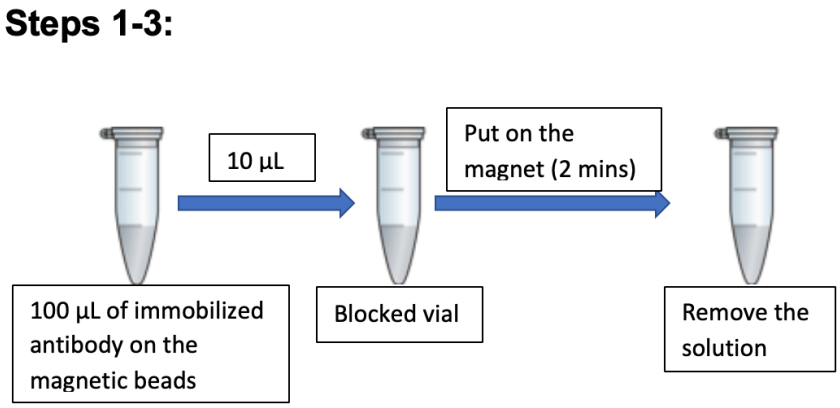

Figure 2.4 – Illustration of steps 1, 2, and 3. This illustration involves the part before adding the surrogate beads into the system.

4. Add the 1 milliliter of pre-prepared surrogate beads with a concentration of 1e9/ml/.

5. Incubate the surrogate beads that are dispensed onto the magnetic beads by using the mixer. Note: The incubation time is one of the variables in our work and varies between 15 minutes to 60 minutes.

6. After incubation, take the centrifuge tube out from the mixer and place it on a magnet for 2 minutes. Allow the beads to collect at the tube wall and remove the supernatant.

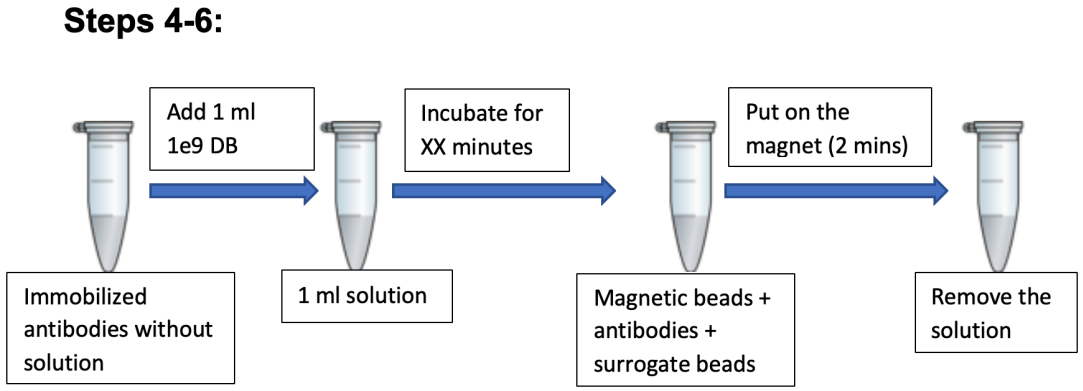

Figure 2.5 – Illustration of steps 4, 5, and 6. This illustration involves the surrogate bead bonding to the immobilized antibodies.

7. Add 1 milliliter of 1% BSA wash buffer. This step is the first washing step.

8. Place the tube onto the mixer and rotate it for 2 minutes to allow magnetic beads to be mixed and become uniform in the wash buffer.

9. After incubation, take the centrifuge tube out from the mixer and place it on a magnet for 2 minutes. Allow the beads to collect at the tube wall and remove the supernatant.

10. Repeat step 8, 9, and 10 two more times to perform three times wash.

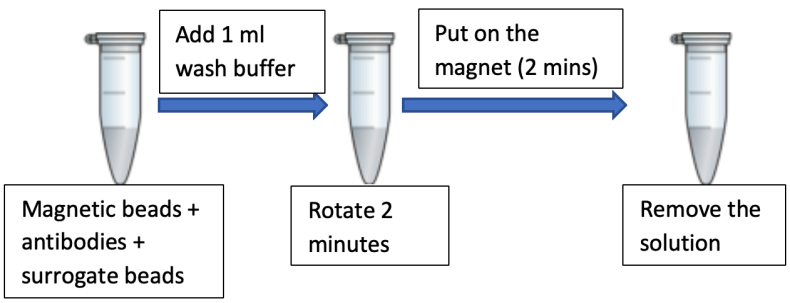

Figure 2.6 – Illustration of steps 7, 8, 9, and 10. This illustration involves the three times wash to remove unbounded particles.

11. Add 1 milliliter of 1% BSA wash buffer and rotate the tube for 2 minutes.

Note: Prepare new blocked centrifuge tubes while rotating the tube for mixing.

12. Aspirate the mixed uniform solution and dispense it into the new blocked vial.

13. Place the tube on a magnet for 2 minutes and allow the beads to collect at the tube wall. Remove the supernatant. Remove the tube from the magnet stand.

14. Add 1 milliliter of elution buffer. Details of the elution buffer were discussed in the Section 2.1.1

15. Incubate the beads and elution buffer by using the mixer for 12 minutes. This step is the step where the surrogate beads separate from the magnetic bead – antibody complex.

Note: Prepare new blocked centrifuge tubes while rotating the tube for mixing.

16. Take the centrifuge tube out from the mixer and place it on a magnet for 2 minutes. Allow the beads to collect at the tube wall and remove the eluted buffer. Dispense it into the new blocked vial.

The main logic to perform steps 11-16 is similar to the previous steps and can be followed through Figure 2.3, 2.4, and 2.5.

2.4 Fluorescence Imaging of the Converted Surrogate Beads

Fluorescence imaging is the visualization of the fluorescence beads for molecular structures [22].Fluorescence sandwich assays have been commonly used to detect target proteins [23]. In our work, we have removed the target protein and sandwiched the antibody between the magnetic bead and surrogate bead. To observe the final result, we have used a Nikon Eclipse TiE series fluorescence microscope.

Three microliters of eluted buffer were taken from the final centrifuge tube and placed onto the glass slide. Blue fluorescent light is used for excitation. 10x objective was used to quantify the surrogate beads (fluorescence) in the sample.

2.5 Automation of the Surrogate Bead Conversion

Researchers have been manually performing immunoprecipitation-based and similar experiments for decades. However, there is a significant need for automation for this type of experiments. Automation of the protocol will also increase the amount of the data obtained within a given time. Hence, we implemented our two-particle sandwich assay design using a pipetting robot and had a successful result.

To achieve an accomplished automation process, we have chosen Opentrons OT-2 pipetting robot. Opentrons OT-2 robot makes pipetting easy and accurate with its single/multi-channel pipette options, and different capacity pipette options. Opentrons OT-2 also is a modular system with add-ons. We have put together Opentrons MagDeck with OT-2 robot to make magnetic bead manipulation possible.

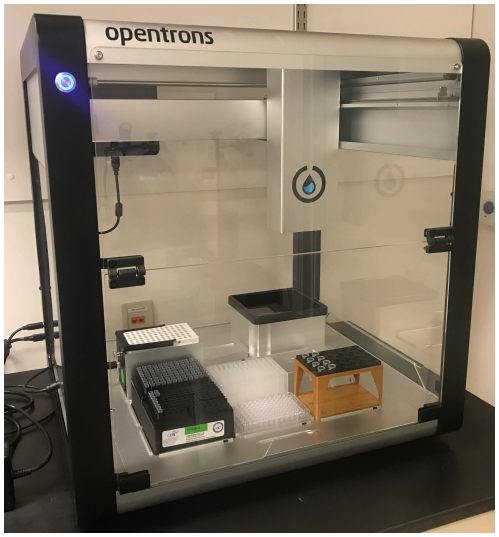

Figure 2.7 – Opentrons OT-2 Pipetting robot with the labware on it. It has single use pipette tip rack, vial holder rack (designed and printed by UCSC Nanoengineering Group), MagDeck for magnetic bead manipulation and 96-well plates on it.

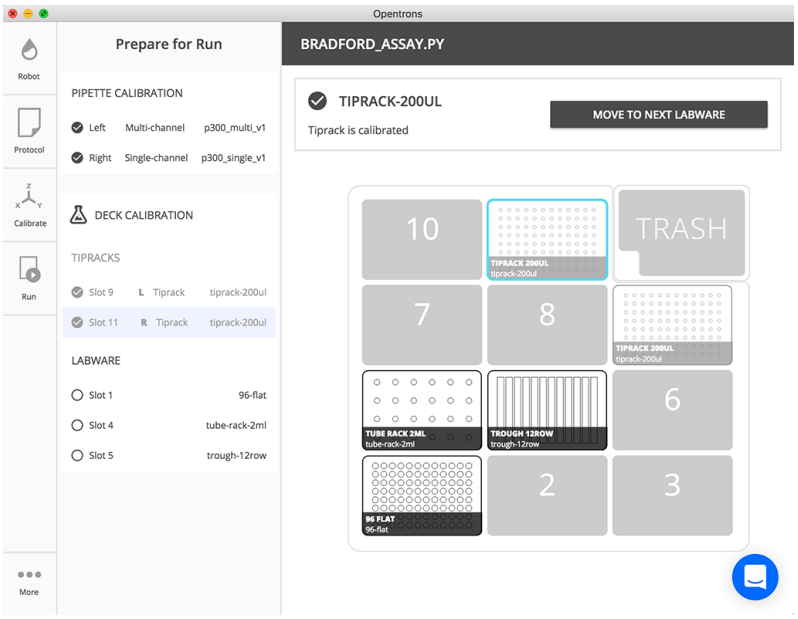

Figure 2.8 – Opentrons App allows flexible design of the labware, pipette types and slot usage. Adapted from [24].

We have used a 96-well plate to fill our reagents. A photograph of our robotic setup is shown in Figure 2.7. Opentrons OT-2 robot has a Python-based user-friendly software to implement custom protocols, as shown in Figure 2.8. The layout of the robot is very flexible and open. Necessary labware can be placed into the robot and modified for the protocol's requirement.

We have developed a script to perform our design with a robot. After the researcher places the reagents in the wells, the robot simply runs the protocol. Part of the developed script is available in the Appendix section.

Chapter 3: Results

3.1 Effect of the Amount of Immobilized Antibody

Although there is an efficiency graph provided by the supplier for the antibody amount per magnetic bead (mg) was shown in Figure 1.1, we have started our experiments by benchmarking the chart, since we are using surrogate beads to characterize immobilized antibody mass.

We have prepared four different concentrations of antibodies for one milligram of magnetic beads. Our antibody amounts were 4, 7, 10, and 13 micrograms. We have chosen these parameters since the efficiency graph shows us that these are good initial estimate. Captured images of the four different concentrations from lower to higher is shown in Figure 3.1. The full-size photos could be seen in Appendix A.

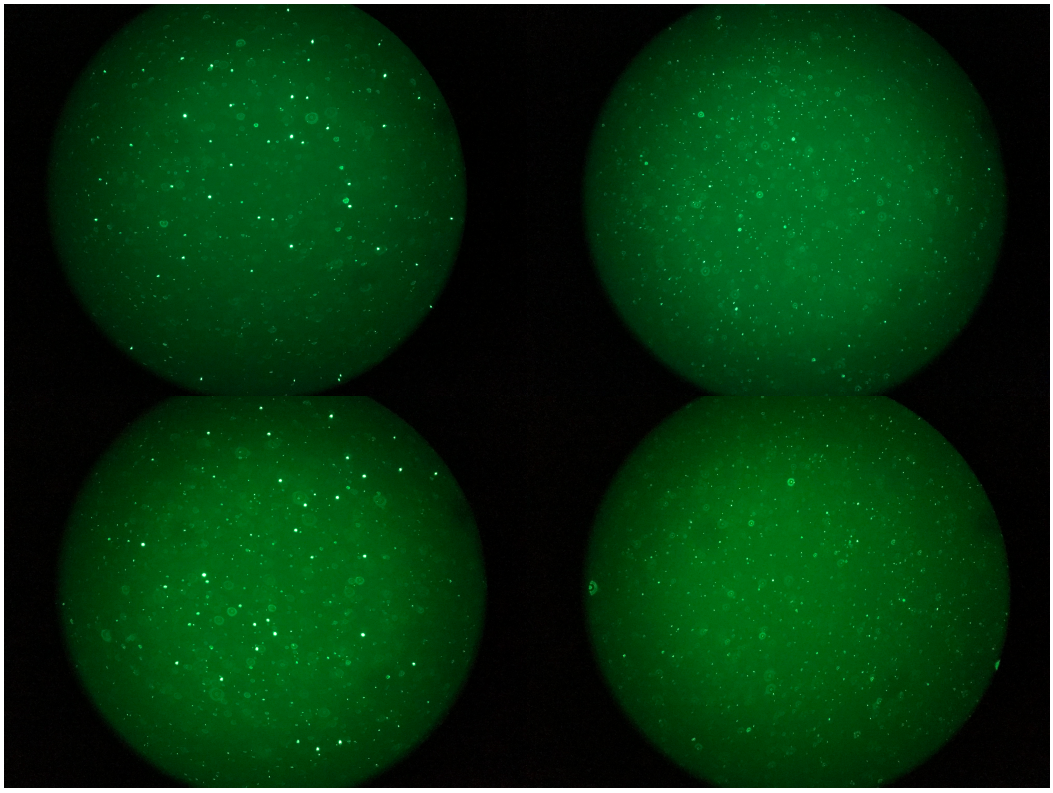

Figure 3.1 – Conversion result for the different amount of antibodies used for the immobilization. Top-left) 4 micrograms. Top-right) 7 micrograms. Bottom-left) 10 micrograms. Bottom-right) 13 micrograms. There is an increasing trend in the number of converted beads from 4 micrograms to 7 micrograms and 7 micrograms to 10 micrograms. However, the number of converted beads tend to decrease after 10 micrograms as expected. Full-size images could be seen in Appendix A.

Droplet samples taken for result observation from the eluted buffer have a volume of three microliters. By using this information and the size of total converted beads (1 milliliter), we have estimated the number of total converted beads for each incubation time, as shown in Figure 3.2.

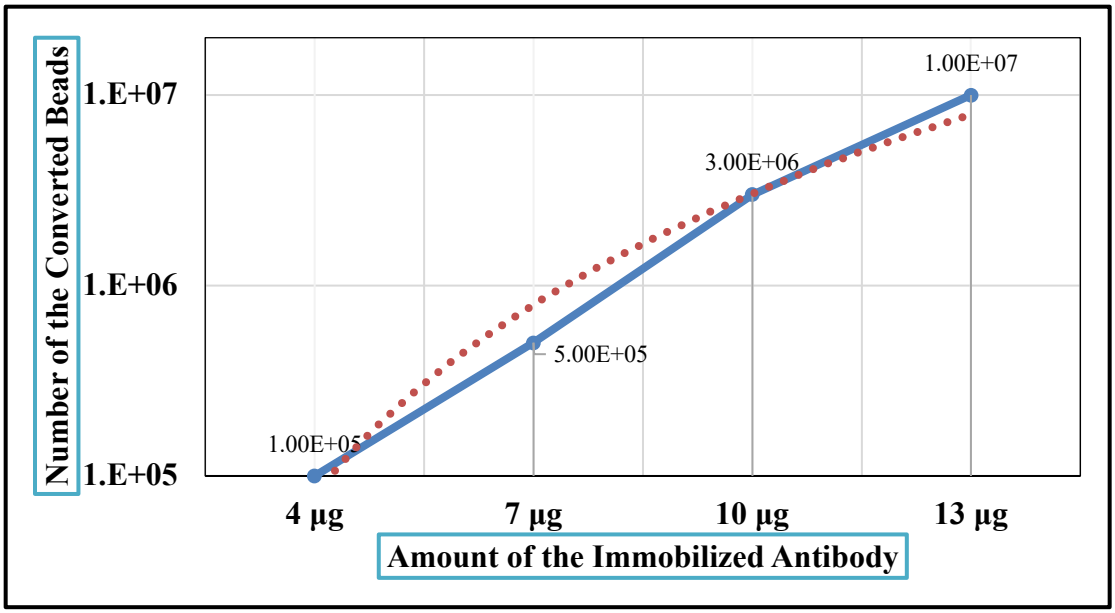

Figure 3.2 – Estimated number of the converted beads for different amount of the immobilized antibodies. Dashed line interprets the saturation expectation as the amount of antibody immobilization increases, and shows similar profile with the data taken from [2].

3.2 Effect of the Incubation Time

In this section, we have tested the incubation time for the surrogate beads to bond them onto the immobilized antibodies. Same experimental conditions applied as 3.1. We have used ten micrograms of antibodies immobilized on 1 milligram of magnetic beads. The incubation times are tested as follows: 15 minutes, 30 minutes, 45 minutes, and 1 hour.

According to the results, we observed that there is a rapid increase in the number of converted beads from 15 minutes incubation to 30 minutes incubation. Forty-five minutes incubation also resulted in a higher number of converted beads. However, 1-hour incubation did not help much to increase the number of converted beads anymore. Still, 1-hour incubation could be applied if time is not a

consideration. Pictures for the four different incubation time conditions are shown in Figure 3.2. The full-size images could be seen in Appendix A.

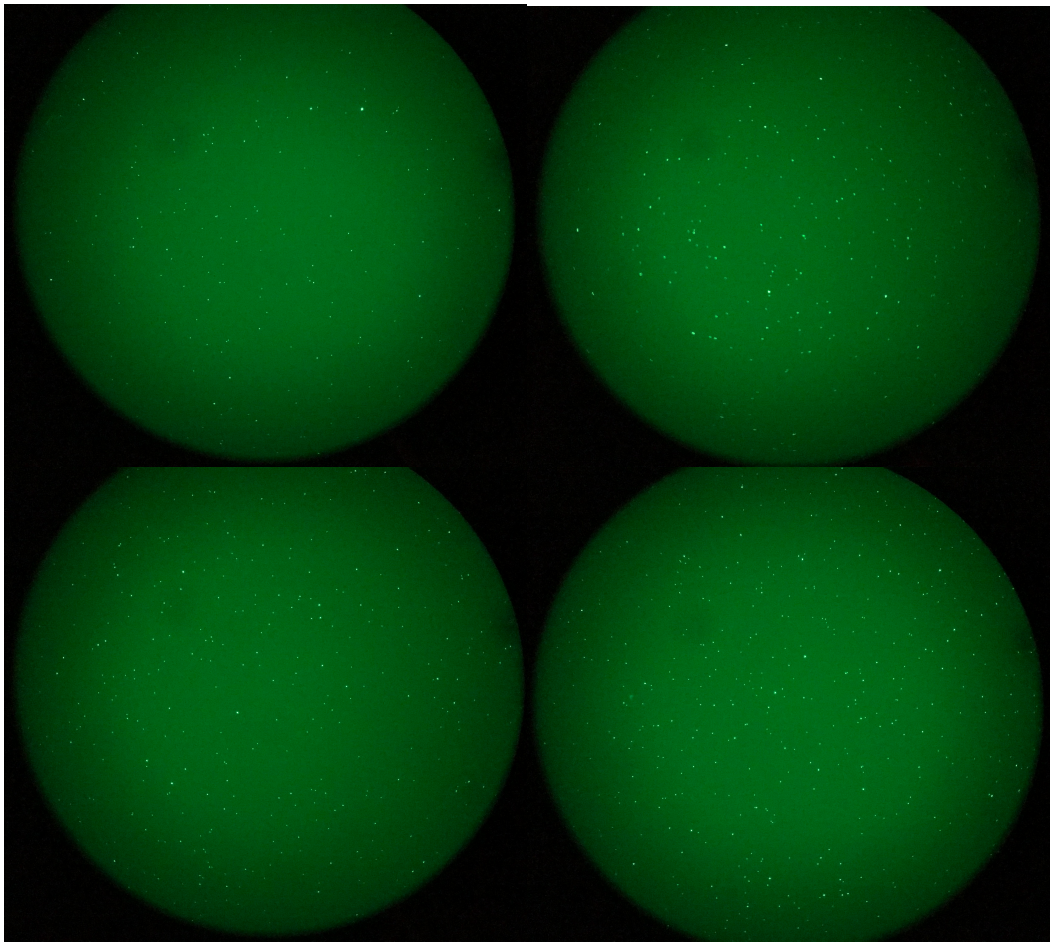

Figure 3.3 – Conversion result for the different incubation times. Top-left) 15 minutes. Top-right) 30 minutes. Bottom-left) 45 minutes. Bottom-right) 1-hour incubation. There is a clear increasing trend in the number of converted beads as the incubation time increases. Full-size images could be seen under Appendix A.

3.3 Deterioration During the Shelf Storage

In sections 3.1 and 3.2, we optimized the incubation time and the amount of antibody used for immobilization. Considering the amount of antibody used and incubation times, we determined that 10 µg antibody provides the optimal conditions for our protocol. 45 minutes of the incubation time gives an excellent performance of the bead conversion, and less time-consuming.

We have tested effect of shelf storage on the antibody immobilization by using the parameters that are used above. Independently from with these considerations, all the immobilized beads were stored at 2 ºC – 6 ºC in a dark environment. Dark environment is need to prevent degradation of the fluorescent molecules loaded within the dielectric beads. Surrogate beads lose their fluorescence characteristics when exposed to the light for a long time [25].

We have tested three different shelf storage time: one day, three days, and six days. We observed that the most successful bead conversion was achieved using 1-day stored immobilized beads. Although 1-day stored immobilized beads gave the best performance, we were able to use that immobilized beads up to one week without significant loss. The converted bead sample images are shown in Figure 3.4.

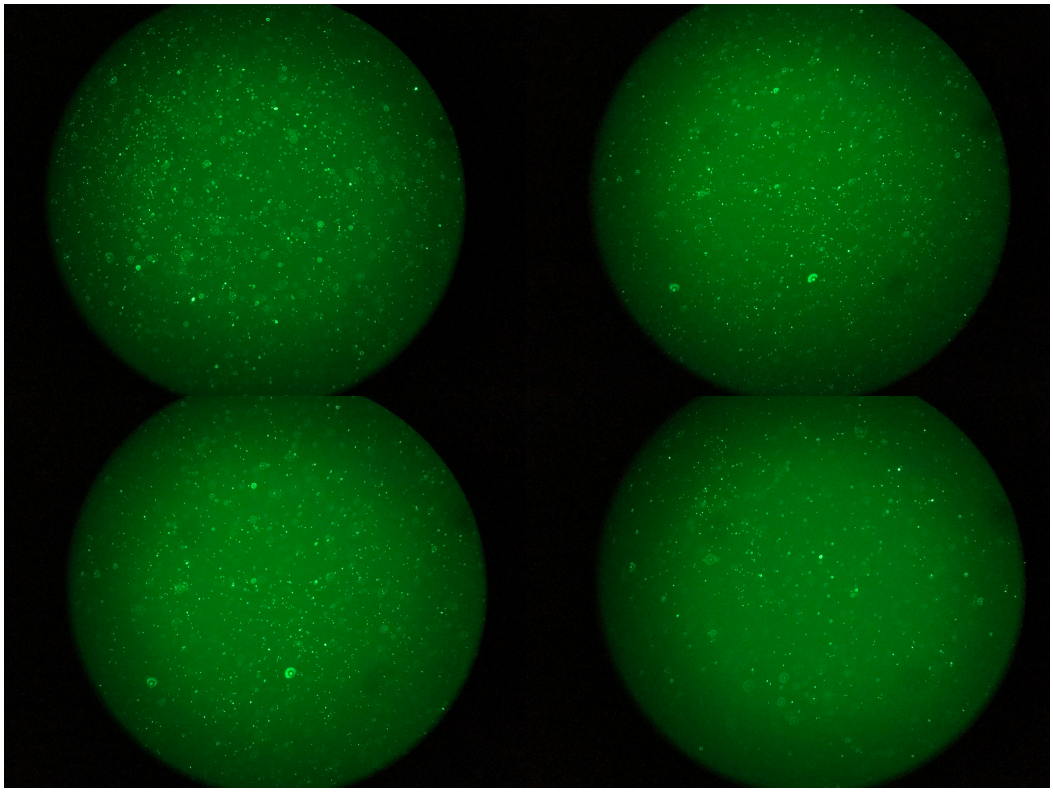

Figure 3.4 – Conversion result for the different shelf storage times. Top-left) Three days later for 4µg antibody coupled beads. Top-right) Three days later for 7µg antibody coupled beads. Bottom-left) Six days later for 4µg antibody coupled beads. Bottom-right) Six days later for 7µg antibody coupled beads. There is a clear drop in the number of converted beads as the shelf storage time increases.

3.4 Conversion Results with the Robot

Bead conversion by using an automated system was one of our primary focus. For these an experiment, we needed a base control sample, called as the negative control, to make sure the conversion in the actual sample performed successfully. For this purpose, we have prepared two different magnetic beads. One has biotinylated antibodies immobilized on them while the other one is merely magnetic beads. Then,

surrogate beads are added into the medium and incubated by the optimized time obtained in Section 3.2.

Our tests showed that robotic scheme was successful in implementing our conversion process. Results of our conversion test are shown in Figure 3.5. The negative control sample showed few surrogate beads as converted, which means we have had non-specific binding. Non-specific bindings caused background signal. The system can be optimized, and the wells can be coated beforehand to decrease the background signal for the real immunoprecipitation applications. The script code used for the robotic protocol is provided in Appendix B.

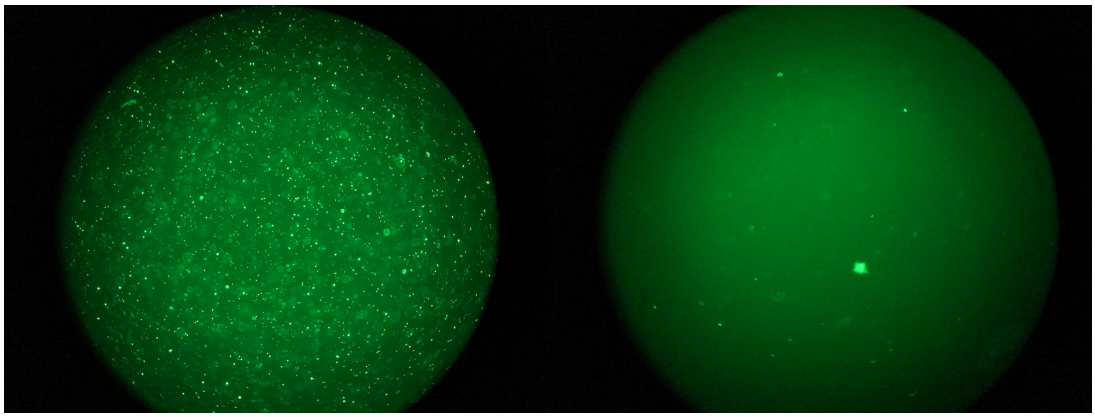

Figure 3.5 – Conversion result of the two-particle complex by using the automated robotic system. Left) Magnetic beads with immobilized antibodies on them was used. Right) Magnetic beads without any capturing ligand on them. There is a clear difference between the results. Negative control has shown some background signal due to non-specific binding.

Chapter 4: Conclusion

We have developed a two-particle sandwich assay complex to measure the efficiency of the antibody immobilization. We have successfully benchmarked the effect of the antibody amount on immobilization protocol given in [2]. We have achieved similar results to Thermo Fisher Scientific's DynaBeads® Antibody Coupling Kit Manual.

In addition to benchmarking, we have tested the effect of incubation time on our sandwich assay complex by binding the surrogates. We have tried five different incubation times to find the optimal incubation time. We have concluded that 45 minutes would be optimal for similar protocols.

We have also tested the shelf lifetime of the immobilized antibodies. We have concluded that it is best to use immobilized beads is one day after the immobilization protocol is completed. However, it is possible to use coupled antibodies up to one week.

We automated our protocol for high throughput testing purposes using Opentrons OT-2 robotic pipetting platform. Automated process allows us to save significant time. It also allows us to execute the protocol in more consistent and accurate manner. Automation of our protocol can easily be modified for more complex applications, including immunoprecipitation and ELISA.

Through this study, we have introduced a novel technique for characterization of antibody couplings in an automated and high throughput manner. Immunoprecipitation has a vital role in protein analysis among the researchers.

Although we have successfully developed, tested, and automated our two-particle complex sandwich assay, there are a couple of things that can be improved.

First of all, we have tested our design using one type of antibody and surrogate beads. I believe testing different antibodies and surrogate beads can provide additional knowledge for pushing the research further. The monodisperse magnetic beads were selected from one supplier only, and the polydisperse beads haven't been tested in this study. In my point of view, testing different vendors' products, and also testing the polydisperse magnetic beads for comparison, would increase the understanding of the antibody immobilization phenomena.

Considering all these opportunities, I believe there is still room to advance the immunoprecipitation protocol optimization, and automation.

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Appendix A - Real Size Images for the Converted Buffer Samples

Figure 3.1 images. From Top-left (4 µg) to Bottom-right (13 µg): 4 µg

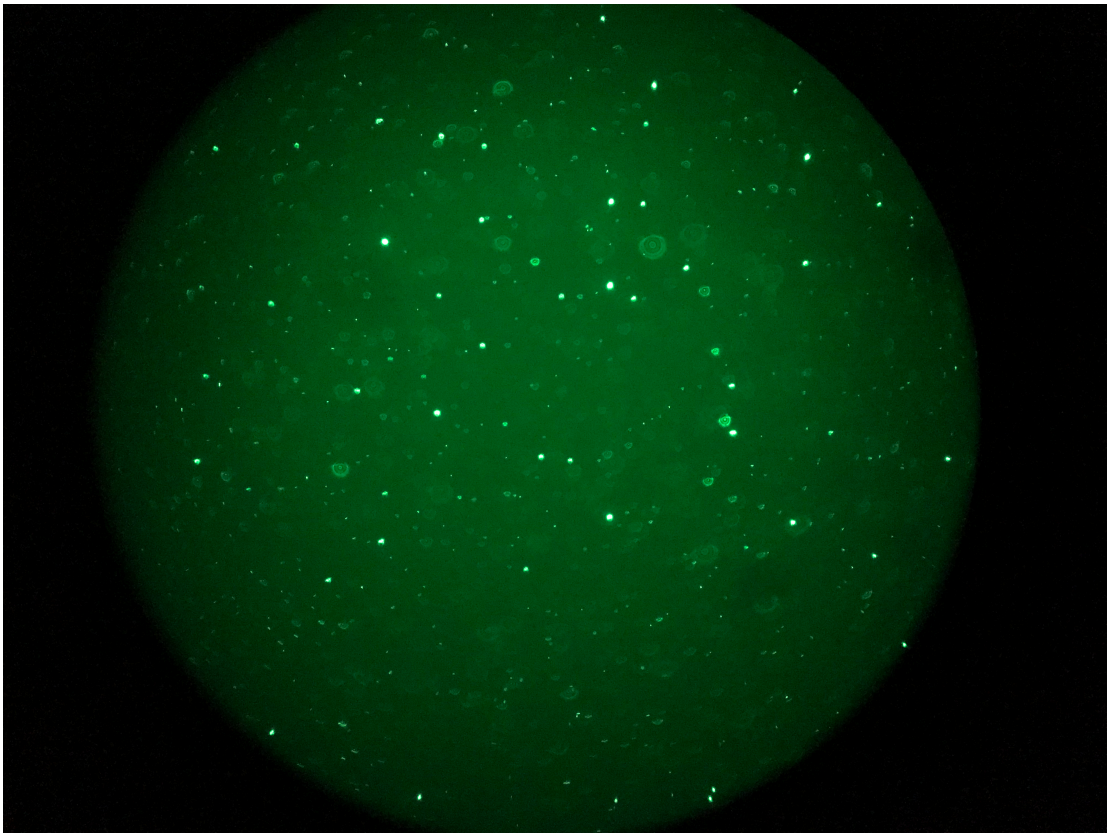


Figure 3.1 images. From Top-left (4 µg) to Bottom-right (13 µg): 7 µg

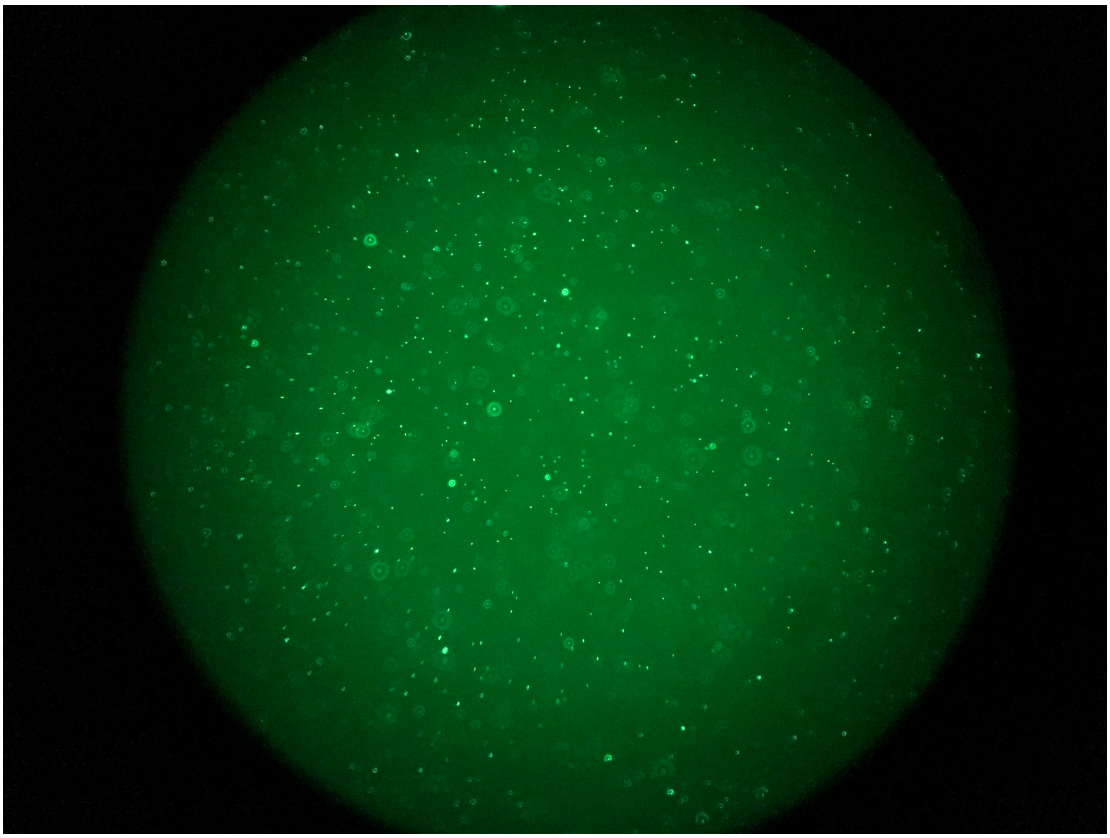


Figure 3.1 images. From Top-left (4 µg) to Bottom-right (13 µg): 10 µg

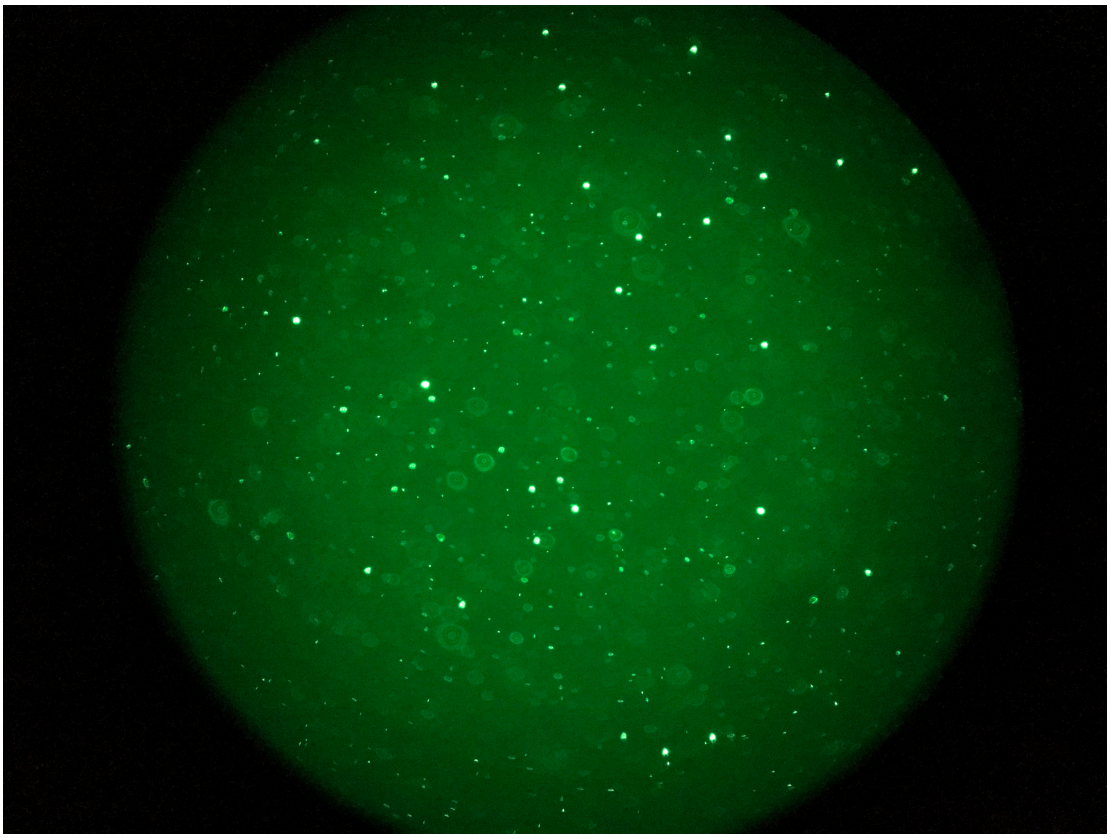


Figure 3.1 images. From Top-left (4 µg) to Bottom-right (13 µg): 13 µg

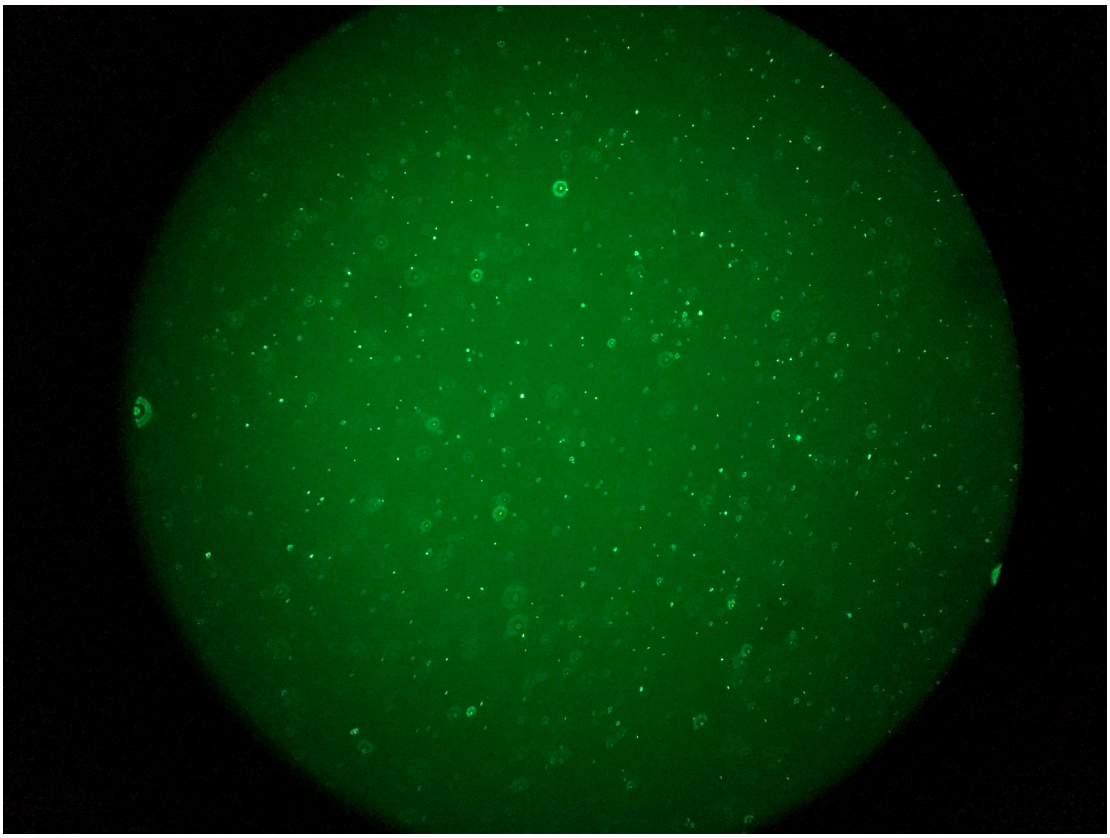


Figure 3.3 images. From Top-left (15 minutes incubation) to Bottom-right (1-hour incubation): 15 minutes.

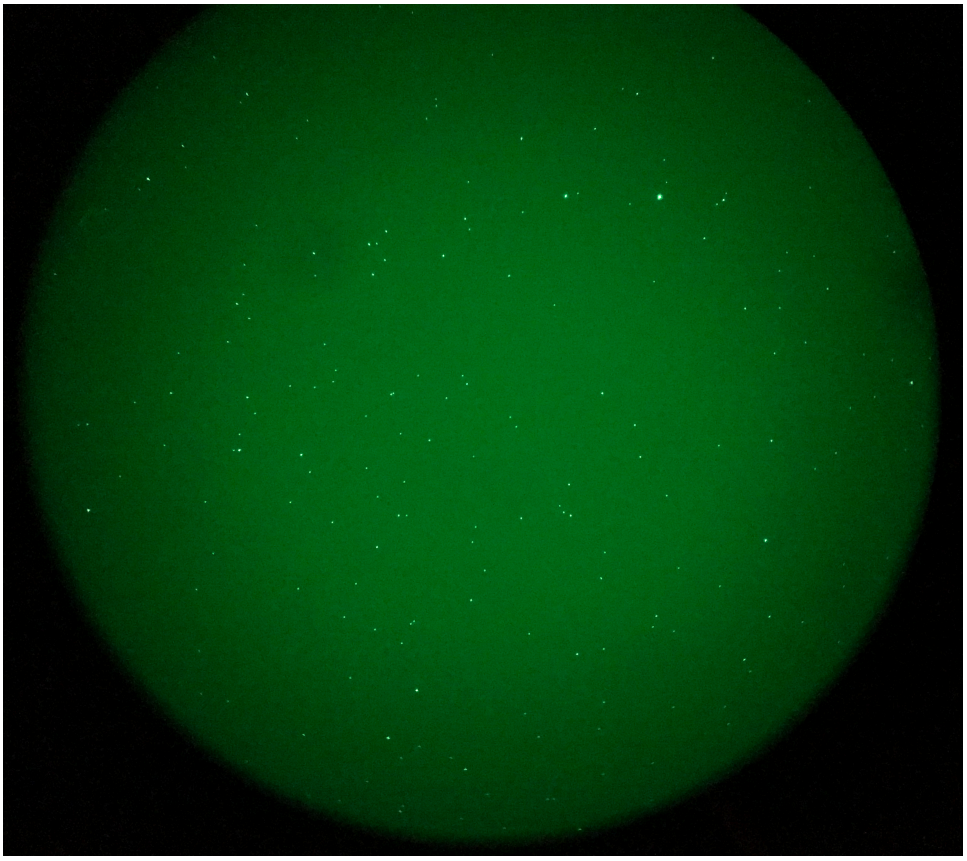
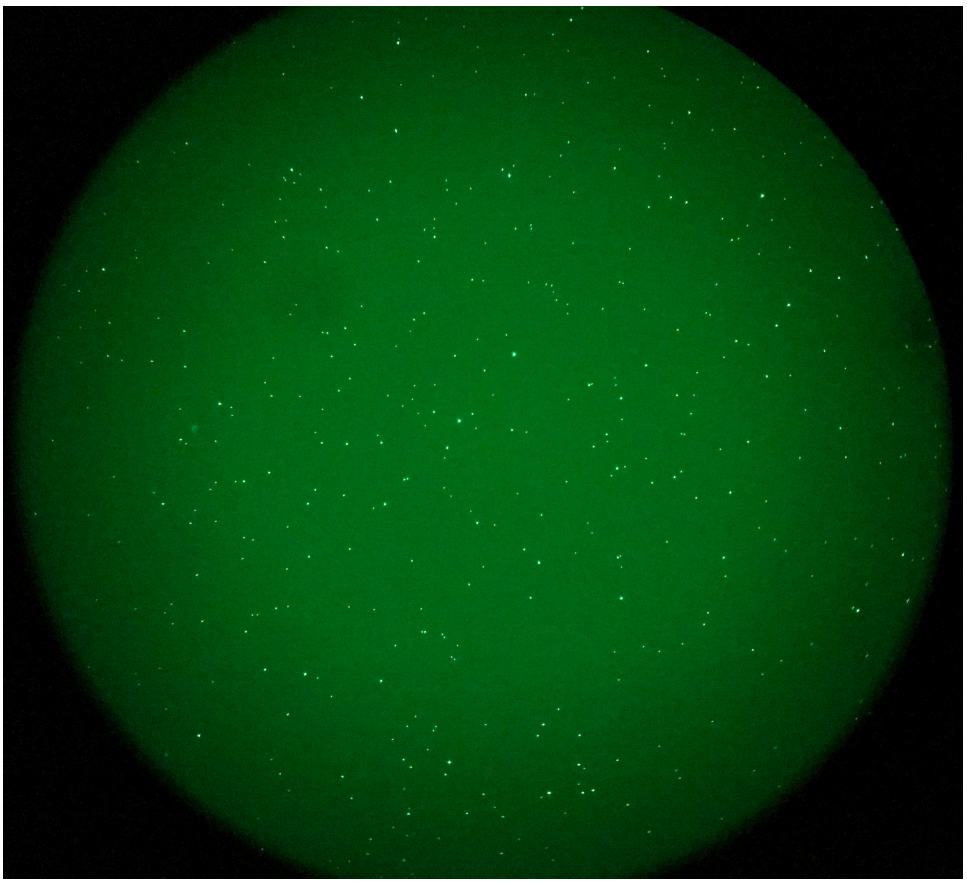


Figure 3.3 images. From Top-left (15 minutes incubation) to Bottom-right (1-hour incubation): 30 minutes.

Figure 3.3 images. From Top-left (15 minutes incubation) to Bottom-right (1-hour incubation): 45 minutes.

Figure 3.3 images. From Top-left (15 minutes incubation) to Bottom-right (1-hour incubation): 1-hour.



Appendix B - Source Script for Automated Protocol

Note: Not all the source code is shared in this work. The shared part gives the general idea to the reader and gives an opportunity for using this script as a base of their protocol.

```
# Used 94 pipette tips, run time is 2 hours 8 minutes

from opentrons import labware, instruments, modules

metadata = {

    'protocolName': 'Coupling Efficiency-300ul-SingleChannel',

    'author': 'Mustafa Mutlu',

    }

# labware setup

trough = labware.load('trough-12row', '1')

mag_module = modules.load('magdeck', '4')

plate = labware.load('biorad-hardshell-96-PCR', '4', share=True)

tiprack_300 = labware.load('opentrons-tiprack-300ul', '2')


# instruments setup

p300 = instruments.P300_Single(

    mount='right',

    tip_racks=[tiprack_300])

# ================================================
        =======

# reagent setup
```

```
# ================================================================
        =======

bsa = trough.wells('A1')

diebead = plate.wells('A2')

elution = plate.wells('A3')

# ================================================================
        =======

# Step 1: Turn on magnet for 30 seconds

# ================================================================
        =======

# turn on magnet for 90 seconds

mag_module.engage()

p300.delay(seconds=90)

# ================================================================
        =======

# Step 2,3: (2), remove supernatant in A1, B1; (3), Drop the tip

# ================================================================
        =======

# discard supernatant in well A1, B1

p300.transfer(15, plate.wells('A1').bottom(0.7), p300.trash_container.top()) # tip is
        already dropped

p300.pick_up_tip()

p300.transfer(15, plate.wells('B1').bottom(0.7), p300.trash_container.top()) # tip is
        already dropped

# ================================================================
        =======
```

```
# Step 4: transferring DBs onto MB (from A2 to A1 and B2 to B1)

#==========================================================
=======

# transfer from A2 to A1

p300.pick_up_tip()

p300.transfer(300, plate.wells('A2'), plate.wells('A1'), new_tip='never') # transfer 300
    ul DB

p300.drop_tip()



p300.pick_up_tip()

p300.transfer(300, plate.wells('B2'), plate.wells('B1'), new_tip='never') # transfer 300
    ul DB

p300.drop_tip()

#==========================================================
=======

# Step 5: Disengaging the magnet

#==========================================================
=======

mag_module.disengage()  # turn off magnet

#==========================================================
=======

# Step 6: Solution mixing (INCUBATION) 20 mins

#==========================================================
=======

# mix 10 times at 300 uL every 1 minutes for 20 times using the same tip

for _ in range(20):
```

```
    p300.pick_up_tip()
    p300.mix(10, 300, plate.wells('A1'))
    p300.blow_out(plate.wells('A1').top())
    p300.drop_tip()
    p300.delay(seconds=30)

    p300.pick_up_tip()
    p300.mix(10, 300, plate.wells('B1'))
    p300.blow_out(plate.wells('B1').top())
    p300.drop_tip()
    p300.delay(seconds=30)

p300.pick_up_tip()
p300.mix(10, 300, plate.wells('A1'))
p300.blow_out(plate.wells('A1').top())
p300.drop_tip()

p300.pick_up_tip()
p300.mix(10, 300, plate.wells('B1'))
p300.blow_out(plate.wells('B1').top())
p300.drop_tip()
#==============================================================
=======
```