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The Basic Guide for re-suspending magnetic beads

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Chapter 10

The Four Main Limitations of using the Sonication Method in Magnetic Bead Suspensions

Chapter 1

Why is magnetic bead resuspension important?

The major problem with non-colloidal solids suspended in liquids is that they will eventually sediment and clump over time. Magnetic bead suspensions are no exception. Even though magnetic separation is generally gentler than separation by centrifugation, clumps and aggregates can still form. Aggregate formation happens at a much greater frequency if you do not use homogenous biomagnetic separation conditions. When aggregates form the full reactive surface area on the aggregated beads is not exposed during the subsequent coupling and coating steps. When this happens, the consistency of the lot is compromised. The problem is amplified when large production batches need to be divided into small volumes. If there is a significant amount of aggregation, there will be very little consistency between the aliquots.

During the process of coating magnetic beads for use in diagnostic kits, there are several steps in which the solid beads need to be separated from the liquid, washed to remove excess antibodies or other biological coating and then resuspended with new buffers. Every time you separate the beads, you must resuspend them completely or functional consistency of the final bead product will be greatly compromised. This is the key to in-lot consistency. A well-homogenized, non-aggregated suspension will lead to consistent aliquots derived from the larger production volume. Bad resuspension processes lead to high in-lot variability.

The resuspension of magnetic beads is comprised of two different processes:

1. Homogenization of the bead suspension. This entails mixing the suspension such that the bead concentration at any given point in the volume of the liquid is the same. Homogenization is always a necessary technique after each bead/liquid separation step in the production process.

2. Deagglomeration, or disaggregation, of the aggregated magnetic beads. This entails various harsher methods of separating beads that are aggregated, sometimes irreversibly. Sonication is one common method used to help separate aggregated beads.

Deagglomeration is only necessary if bead aggregates are formed due to excessive magnetic force during the separation process or if aggregates are formed due to a lack of adequate blocking of the bead surface.

Because of the possible difficulties dealing with aggregated beads, it is vitally important to pay close attention to the proper methods of resuspension. A clear understanding of the key principles of resuspension and how new technologies can alleviate or avoid aggregation will result in more consistent product.

Chapter 2:

The Five Most Used Resuspension Methods: What Works and What Does Not Work

Resuspension of the magnetic beads is the key to guaranteeing both in lot and lot to lot consistency of the resulting *in vitro* diagnostic (IVD) magnetic bead reagents. In order to achieve resuspension of solids with liquids, scientists and technicians in the lab typically use five different general techniques:

1. Vortexing
Vortexing is normally used when one has a small tube with the reagent to be resuspended. They hold the tube against a rubber surface that is shaking at varying speeds and the content of the tube will shake as well. This can only be used with small tubes.

2. Roller bottle mixing
This technique can be used with much higher volumes. People often put these large volumes in a cylindrical bottle and place them on an assembly consisting of rubber rolling cylinders.Scientists can maintain their bead suspension sometimes for years in this manner.

3. Overhead mixing
This technique is used with large bottles and bioreactors. An agitator is placed through the top of the bottle into the liquid and a motor continually moves the agitator to keep the beads in solution. This is a very popular technique to use with bioreactors.

4. Magnetic stirrer
Another way to maintain a suspension or resuspend solids is to place a magnetic bar in the solution and place the bottle or flask on top of a stirring device. The stirring device also generates a magnetic field that rotates at varying speed and will cause the bar inside the liquid to spin at the same speed.

5. Sonication

The first four methods are mixing and homogenization methods, but none of them generate enough energy to pull aggregate beads apart from each other. Sonication, although complex and difficult to master at large volumes, can break the aggregates apart.

Since this technology works with magnetic beads, scientists and technicians are forced to use non-magnetic materials for any resuspension method of choice. Magnetic materials are not used in vortexing and rolling equipment. However overhead mixers and sonicators sometimes include materials that are magnetic. This issue can be addressed by using non-magnetic stainless steel or other material that does not interfere with magnetic bead separation.

Magnetic stirring, by its very nature, should be discarded as a viable process. The stirrer used in the process is usually a ferrite magnet or some other magnetic material that would attract the magnetic beads and ultimately cause the formation of clumps and aggregates. A certain percentage of magnetic beads and their attached biomolecules remains attached to the stirrer generating variable losses from batch to batch that are difficult to quantify. Moreover, the intense external magnetic fields necessary to rotate the stir bar exposes the beads to additional uncontrolled dynamics making it even more difficult to parameterize and validate production processes.

Therefore, eliminating magnetic stirring as a viable option for homogenizing magnetic bead suspensions leaves the scientist with four other homogenizing options listed in the text.

Chapter 3:

The Two Questions You Must Ask Before Using the Sonication Method with Magnetic Beads

Sonication is used to break up irreversible magnetic bead aggregates when they are generated during various steps of the production process. However, sonicating a large volume is not a straightforward process. To set up a large volume sonication, you must choose the proper probe, the power, and the conditions, but choosing and determining these things can be tricky.

One thing you must question is **'Why do I have aggregates in the first place?'**
There are two possible causes of irreversible aggregation that you need to investigate:

1. Did you properly block the exposed hydrophilic bead surface after coating?

During the production process, you will coat the beads, but you will not always be able to coat the entire surface of the beads. If you have not blocked the parts of the beads not covered by your antibodies or antigen, these exposed surfaces can react with other proteins or with the protein on neighboring beads. If you are using the correct blocking reagent, you will reduce auto-aggregation, thereby reducing non-specific background reactions. Since avoiding aggregate formation altogether greatly simplifies re-suspension, it is worth trying different blocking reagents to find the optimal re-suspension conditions (e.g. BSA, Tween 20, Triton-X 100, etc.).

2. Are you applying excessive magnetic force when separating the beads?

The magnetic force in classical separation systems decreases quickly with distance from the magnets. Because of this, technicians need to generate extremely high forces in the magnetic bead retention areas in order to have enough force to capture beads far from the magnets. In addition, since the forces are weak far from the magnets, separation times are long, causing the beads in the retention area to experience high magnetic

forces over long periods of time. High magnetic forces can cause irreversible bead aggregation in spite of optimal blocking techniques.

Modern homogeneous biomagnetic separation systems, however, generate the same force in the entire working volume, thus decreasing the time for bead recovery, increasing the amount of beads recovered and decreasing the forces the beads experience in the retention area over time. Gentler retention forces, larger retention areas and the shorter exposure time to magnetic forces can result in an avoidance of irreversible aggregation altogether.

If you can avoid irreversible aggregation, mixing via homogenization is the only technique necessary to resuspend the beads allowing for a simpler, more predictable process that is easy to scale up.

Chapter 4:

Three Questions to Help Decide What Technique to Use for Magnetic Bead Mixing and Homogenizing

Magnetic beads need to be constantly mixed and homogenized to avoid sedimentation and clumping problems. Even when sonication is necessary to break up aggregates, mixing and homogenization is necessary. The selection of a specific mixing technique for each step of the production process depends on many factors, but mainly depends on empirical data on the particular process and on any requirements imposed by the properties of the vessel used for the end user's experiments. In addition, it is advisable not to change vessels with each step.

To determine which technique and vessel is best, we can ask the end user the following questions:

1. Are you able to close the bottle or vessel you are using for resuspension and guarantee that the vessel is completely sealed?
If the answer is yes, then the easiest mixing method to use is a roller table. Roller tables are easy to use and easy to scale up. The main drawback of roller bottles is that if there is any problem with the bottle cap, leakage and loss of material will result.

2. Do your bottles need to remain open during your process?
If yes, you can choose from a couple of options depending on your team's experience.

- **You can use a tilted roller mixer.** With this technique there is no direct contact with the suspension. You would need to determine the correct angle of the bottle, the rotation speed, and the maximum filling factor.

- **You can use an overhead mixer.** This apparatus contacts the suspension, but people typically have a lot of experience with this technique. This type of mixer is common in bioreactors and other apparatus. Of course, you need to use non-magnetic materials for the shaft and blade. With this technique the potential for contamination is a common consideration.

3. Do you need to use a fixed vessel or bioreactor for your process?
This scenario is true especially when volumes are scaled up to large volumes. In this case, try to take advantage of overhead mixers that are included in the bioreactor, as long as the shaft and blades are not magnetic .

Before you even begin your process, it is important to understand what resources you have, what experience your team has and how you will ensure that your magnetic biobeads are most efficiently and effectively resuspended. The choice between closed and open bottles, roller mixers, overhead mixers and bioreactors will depend on your unique process and experience.

Chapter 5:

The Two Most Popular Magnetic Bead Mixing and Homogenizing Technologies

Mixing and homogenizing a magnetic bead suspension correctly after each step of coating and washing is the key to maintaining in-lot consistency. During the production process, the beads are resuspended several times in different buffers either while being coated with biomolecules, while capturing biomolecules or during washes. The beads should be homogenized completely in these buffers such that each small aliquot from the larger volume will be the same functionally. It is important that each aliquot have the same reactivity as any other aliquot from the same batch.

Magnetic beads will drop out of solution and sediment over time. Because of this, magnetic beads should be mixed continuously. Mixing should take place even if aggregates are already present in the mixture. For small volumes (up to a few ml), a vortex agitator is a mixing option. Once you increase the volume and cannot use a vortexer, you theoretically have three options for homogenization: magnetic stirrers, rolling mixers and overhead mixers.

It is important to note that magnetic stirring is contraindicated for magnetic beads since the magnetized material would stick to the magnetic bars and be affected by the magnetic field generated by the stirring platform. The two mixing options that remain are rolling mixers and overhead mixers.

Rolling mixers accommodate cylindrical bottles that are typically lying on their sides (or horizontal). If you must have the bottle opened for your process, a tilted rolling mixer would be appropriate in order to minimize contact with the solution and minimize contamination.

An overhead mixer is the popular option when bioreactors are used. For an overhead mixer you will need to choose a shaft and blade that are not magnetic. Be very careful when choosing. Some grades of stainless steel are magnetic and even those grades rated as non-magnetic can be magnetized if they are not properly annealed after machining. Overhead mixing vessels can either be fixed in place or can be moved depending on the apparatus.

Once again, the choice of homogenization technology will be dependent on what is available for your use, your own experience and the nature of your process. Maintaining a good suspension with the appropriate technique will ultimately result in highly consistent results.

Chapter 6:

Mixing and Homogenizing Magnetic Beads without Touching the Suspension

One of the biggest worries of scientists and technicians in a lab is contamination. Avoiding contamination is best accomplished by using closed mixing vessels (normally closed roller bottles). Closed vessels allow constant mixing and homogenizing without worrying about contacting the suspension. Roller mixing can also be easily scaled up to tens of liters.

Contamination can also be a problem if beads are stored for long periods of time. During this time in storage it is important that beads are constantly mixed and homogenized in order to avoid the sedimentation and aggregation that can increase variability and adversely affect in-lot consistency. Because of these potential difficultiess, closed roller bottles and roller mixers are commonly used for long term storage of magnetic beads.

The main problem of using closed bottles is that buffers need to be changed many times during the production process. During washing steps in biomagnetic separation, for example, the liquid needs to be manually extracted and replaced with new buffer several times. If mixing and homogenizing processes are performed using roller mixers requiring closed bottles the vessels must be closed tightly after each separation step. If the caps are too loose, or if the seal is compromised in any way, leakage can occur causing a loss of material and variability of the resulting product.

If roller mixers are still the homogenization method of choice, even with the above limitations, there is a way to use them with open bottles. The vessels must be placed in a near vertical position and then tilted for turning. If used in this way, the bottles should not be completely filled in order to avoid spills and contamination. The angle of tilt and the turning speed can be adjusted to guarantee optimal homogenization of the suspension. If the technique is done properly and care is taken to protect the beads and the liquid, one can easily change buffers and maintain the magnetic beads in a homogenized state with little difficulty .

Contamination should be a constant focus and concern when homogenizing magnetic beads or keeping magnetic beads in suspension over long periods of time. Although closed bottles are good protection against contamination during homogenization,

constant opening and closing the bottles between wash steps can introduce contamination as well. Using open roller bottles on a tilted roller table affords the user the ability to homogenize without touching the liquid while not losing any of the solution, as long as the bottle angle and speed of rotation is carefully chosen.

Chapter 7:

Mixing and Homogenizing Magnetic Bead Suspensions in Bioreactors

When the volume of homogenized magnetic beads is so large that the manipulation of any type of vessel would be cumbersome and impractical, researchers and technicians favor bioreactors instead of roller bottles to process the beads. In a bioreactor the vessel is fixed and therefore a roller mixer cannot be used. The option used for mixing and homogenizing the beads in a bioreactor is to use an overhead mixer.

Overhead mixers are in large demand in biotechnology mainly because of the need to grow large populations of cells in bioreactors. When cell cultures are grown in this manner, they need to be homogenized for optimal growth conditions and so overhead mixers have been developed over the years to deal with these processes with minimal contamination. The advances in overhead mixer and bioreactor technology can also be applied to magnetic bead suspension processes.

An overhead mixer is comprised of an electrical or air-driven motor attached to a shaft with an agitator blade on the end. The rotation speed of the shaft and blade can be varied depending on the volume and suspension characteristics (i.e. bead density, viscosity of the buffer and concentration).

Overhead mixers are not the most desired choice of technicians for the homogenization of magnetic beads for three main reasons:

1. **The risk of contamination from outside particles and from mechanical wear is great even if the apparatus uses sealed rings.** There is also fear that as the suspension is mixed by the rotating blades, particles from outside the vessel or from the wearing of mechanical parts can drop into the vessel.
2. **Depending on the shape of the blades, if they are not well-designed, some researchers are concerned that the blades may damage the magnetic beads or even the attached ligands.**
3. **You must avoid using magnetic materials in the blades and shaft.** Typically the materials that are commonly used for the shaft and blade such as stainless steel can have residual magnetism if the parts are not carefully machined and so this is a concern.

Similar concerns with this method are experienced in other areas of biotechnology and

in upstream processes. These potential problems can dramatically affect production results. The good news is that these problems and concerns have been successfully addressed in other biotechnological processes. The fact is even with these concerns, overhead mixing in a bioreactor is the best option if the vessel is fixed and if large volumes need to be homogenized.

Chapter 8:

When Optimal Disaggregation of Magnetic Bead Aggregates is Necessary

Irreversible aggregation is one of the main concerns when functionalizing magnetic beads for use in diagnostic and biotech applications. The presence of aggregates can cause high variability in the functionality of the magnetic beads and large inconsistencies. Irreversible aggregation causes serious problems when one tries to resuspend the particles after the processing, coupling and washing steps.

Irreversible aggregation of magnetic beads results from two main factors:

1. **Problems with exposure of the beads to high magnetic forces over a long period of time can also cause aggregation, regardless of the coating efficiency.** Excessive magnetic forces on the beads in the retention area may overpower the inherent electrostatic repulsion between the beads. The effect is exacerbated when separation times are long since the first beads to arrive in the retention area will be exposed to high forces over a long period of time.

2. **If researchers do not follow the appropriate steps to avoid irreversible aggregation after each of the processing steps (coating, conjugation and washing), the clumps will ultimately limit the available bead surface and generate functional variability.** Even worse, aggregates will act as larger beads during magnetic separation, completely changing the dynamics of the process. Aggregates will reach the retention area much faster than the calculated time to retention of non-aggregated beads.

If aggregates are not dealt with during the magnetic bead processing steps, the best case scenario is that the variability of the final aliquots will be high. In-lot consistency is greatly compromised. In the worst case scenario, if variability is too high, the entire production lot may need to be discarded, wasting time, effort, resources and money.

If aggregate formation during processing is unavoidable, it is important to include methods such as sonication to break apart the aggregates after each processing step.

Chapter 9:

Five Points to Consider When Using Sonication Methods on Magnetic Beads

Irreversible aggregation of magnetic beads is a great concern among researchers because of the variability it introduces into the process. Knowing how to deal with these aggregates when they do form is the best way to increase in-lot consistency.

Under conditions where hydrophilic bead surfaces are exposed due to inefficient blocking or when excessive magnetic forces are used during magnetic bead separation, the beads are susceptible to irreversible aggregation. It is vitally important to try everything possible to avoid irreversible aggregation by using better blocking reagents and gentler biomagnetic separation techniques. But sometimes the formation of aggregates is unavoidable and the resulting aggregates cannot be resolved with only homogenization techniques (roller mixers or overhead mixers).

Therefore, the attractive forces causing the aggregation must be overcome in some other way in order to disperse the aggregated clump into individual beads. The use of an immersion sonication probe is a good and proven method to gently disaggregate the undesired clumps of magnetic beads.

There are **five things to consider when using sonication to break up aggregates:**

1. The choice of sonication probe is very important and is dependent on the vessel volume and the desired intensity of sonication. The correct probe is the key to optimal performance of the method.
2. The depth to which a probe is immersed is one of the key parameters when considering reproducibility issues.
3. Temperature stability of the suspension during sonication is critical. This is especially important for small volumes. Temperature rise should be monitored since the ultrasonic intensity necessary for disaggregation is much higher than needed for other sonication applications.
4. Consider using repeated short cycles of mixing and sonication in order to avoid both a rise in temperature and sedimentation during the process.
5. The probe must always be kept clean in order to avoid cross-contamination

between processes.

Sonication is not an exact science. However, if the above five points are taken into consideration, sonication is a highly useful tool to efficiently disaggregate magnetic beads formed during the production process.

Chapter 10:

The Four Main Limitations of using the Sonication Method in Magnetic Bead Suspensions

Sonication is a very effective method for breaking up magnetic bead aggregates, but it is not a panacea. It is an efficient technique once all of the parameters are set, but it is a complex technique with some important limitations, especially for high volume processes.

The limitations to consider are:

1. **You must have the sonication probe in contact with the sample.** This necessitates strict control over probe cleaning in order to avoid cross-contamination and contamination in general.
2. **The probe design is important.** If you find that one probe is highly efficient for one type of vessel and one volume and then must scale up or down, you cannot extrapolate probe properties for a different vessel, different volume, different vessel material or different shape vessel. You must design a different probe for each different volume and change in vessel.
3. **Reproducibility of sonication results is challenging.** The transmitted energy is strongly dependent on the probe position, specifically how deeply the probe is immersed. A rise in temperature especially in small volumes can affect reproducibility because it is can cause damage to the bead ligands. Normally in large volumes reproducibility due to temperature extremes is not a problem since there is space for the extra energy-induced heat to dissipate.
4. **The microstreaming effect is limited to the area surrounding the vicinity of the probe.** Therefore repeated cycles of mixing, homogenizing and sonication are required to effectively break up a maximal amount of aggregations.

Despite the above limitations of sonication, this method is an extremely useful technique to break apart aggregates and maintain functionality of the coupled magnetic beads. Of course it is always best to avoid the formation of aggregates altogether, but if aggregation is an unavoidable fact of your process, sonication is an excellent option.