IMMUNOSPHERES®

Bead Method for the Detection of Sperm-Reactive Antibodies

(about 140 determinations)

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

Principle:

The ImmunoSpheres® method can be used to detect the presence or absence of all classes of immunoglobulins on the surface of sperm using latex beads coated with antibodies that bind to human IgA, IgG and IgM antibodies.

In the *Direct* ImmunoSpheres®, live motile sperm are mixed with a suspension of bead reagent. As the sperm swim through the bead suspension, the beads will bind to the sperm if antibodies are present on the sperm.

In the *Indirect* ImmunoSpheres®, live motile sperm are incubated with diluted serum. Any antibodies to sperm present in the serum will bind to the sperm. Then unbound antibodies and serum proteins are washed away.

In the next step, these sperm are mixed with a suspension of bead reagent. The protocol proceeds as in the *Direct* ImmunoSpheres®.

Reagents:

Anti-Ig(H&L) Beads: 0.8 ml white latex beads coated with (goat) anti-human immunoglobulin heavy and light chains in protein buffer with 0.1% sodium azide.

Materials Required But Not Provided:

1. Sperm washing medium containing 1 - 5% bovine serum albumin.
2. Positive and negative serum controls.
3. Centrifuge capable of 1000*g*.
4. 37°C incubator.
5. Conical centrifuge tubes and rack.
6. Pipettors and tips.
7. Glass slides and coverslips.
8. Sperm counting chamber.
9. 56°C incubator.
10. Bright-field microscope with 100X - 400X magnification.
11. Collecting cups.

Storage and Stability:

Store the reagents at 4°C. They can be used until the expiration date on each label.

Anti-Ig(H&L) Beads should be stored in an upright position.

Once Anti-Ig(H&L) Beads have been washed, they can be stored up to 3 days at 4°C. Or, return washed Anti-Ig(H&L) Beads that were suspended in sperm washing medium but not used in the experiment to the original bottles rather than discarding them.

Warning and Precaution:

All semen and serum specimens should be considered potentially infectious. Handle all specimens as if capable of transmitting HIV or hepatitis. Specimens should be disposed of in accordance with OSHA guidelines.

Specimen Collection:

Semen should be collected in a clean cup. The semen sample should be stored at room temperature until use. Semen should be used within three (3) hours of collecting.

Blood should be collected and stored as serum for up to 7 days at 4°C. If storage time exceeds 7 days, frozen storage in a non-defrosting freezer is recommended. Multiple freeze-thaws should be avoided. Allow previously frozen serum samples to thaw and mix completely before use.

Limitations:

Direct ImmunoSpheres®: Sperm with a motility of less than 5 million/ml cannot be used in this test. Indirect ImmunoSpheres®. At least 50 million motile sperm/ml are needed.

Preparation for Direct ImmunoSpheres®:

1. Bring all reagents to room temperature.
2. Warm sperm washing medium to 37°C. *Caution*: Do not use medium containing human serum albumin.
3. Preparation of Anti-Ig(H&L) Beads:
   - 3.1. Gently swirl the vial containing the Anti-Ig(H&L) Beads, avoiding foaming, to resuspend the beads
   - 3.2. Remove an aliquot (use 10 ul for each sample you will be testing) to a conical centrifuge tube.
   - 3.3. Add 2 -3 ml sperm washing medium.
   - 3.4. Centrifuge at 1000*g* for 5 -10 minutes, remove supernatant.
   - 3.5. Add 2 -3 ml sperm washing medium.
   - 3.6. Centrifuge at 1000*g* for 5 -10 minutes, remove supernatant and resuspend bead pellet to its original aliquot volume.
   - 3.7. Store unused washed Anti-Ig(H&L) Beads at 4°C for up to 3 days.
4. Semen preparation:
   - 4.1. Allow semen sample to liquify.
   - 4.2. Add sufficient sperm washing medium to equal twice the volume of the semen sample and mix. For example, for 2 ml semen, add 4 ml sperm washing medium.
   - 4.3. Centrifuge at 600*g* for 5 - 10 minutes, remove supernatant, and resuspend sperm pellet in about 3 ml sperm washing medium.
   - 4.4. Centrifuge at 600*g* for 5 - 10 minutes, remove supernatant, and resuspend sperm pellet in a small volume of sperm washing medium.
   - 4.5. Count sperm and determine motility of washed sperm.
   - 4.6. Dilute sperm to give a final concentration of 10 million

motile sperm/ml.

Procedure for Direct ImmunoSpheres® of Sperm:

1. Pipette 5 ul of the sperm suspension onto a prewarmed glass slide.
2. Pipette 5 ul of the washed Anti-Ig(H&L) Beads onto the sperm suspension. Use the pipette tip to mix the suspension and Anti-Ig(H&L) Beads thoroughly.
3. Place a coverslip on top of the mixture.
4. After 2 - 5 minutes examine the slide using a microscope.
5. Count 100 free-swimming sperm and determine if and where any beads are bound to the surface of the sperm.

Preparation for Indirect ImmunoSpheres® of Serum:

1. Bring all reagents to room temperature.
2. Warm sperm washing medium to 37°C. *Caution:* Do not use medium containing human serum albumin.
3. Preparation of Anti-Ig(H&L) Beads:
   - 3.1. Gently swirl the vial containing the Anti-Ig(H&L) Beads, avoiding foaming, to resuspend the beads.
   - 3.2. Remove an aliquot (use 10 ul for each sample you will be testing) to a conical centrifuge tube.
   - 3.3. Add about 10 ml sperm washing medium.
   - 3.4. Centrifuge at 1000*g* for 5 -10 minutes, remove supernatant.
   - 3.5. Add 2 -3 ml sperm washing medium.
   - 3.6. Centrifuge at 1000*g* for 5 -10 minutes, remove supernatant and resuspend bead pellet to its original aliquot volume.
   - 3.7. Store unused washed Anti-Ig(H&L) Beads at 4°C for up to 3 days.
4. Serum preparation: heat inactivate serum by incubating at 56°C for 30 minutes.
5. Semen preparation:
   - 5.1. Allow semen sample to liquify.
   - 5.2. Add sufficient sperm washing medium to equal twice the volume of the semen sample and mix. For example, for 2 ml semen, add 4 ml sperm washing medium.
   - 5.3. Centrifuge at 600*g* for 5 - 10 minutes, remove supernatant, and resuspend sperm pellet in about 3 ml sperm washing medium.
   - 5.4. Centrifuge at 600*g* for 5 - 10 minutes, remove supernatant, and resuspend sperm pellet in a small volume of sperm washing medium.
   - 5.5. Count sperm and determine motility of washed sperm.
   - 5.6. Dilute sperm to give a final concentration of 50 million motile sperm/ml.

Procedure for Indirect ImmunoSpheres® of Serum:

1. Pipette 50 ul of the following into separate test tubes:
   a known positive control serum,
   a known negative control serum, and
   each unknown serum.
2. Pipette 400 ul of the sperm washing medium into each test tube.
3. Pipette 50 ul of the donor sperm suspension into each test tube. Mix gently. Cover each test tube and incubate 60 minutes at 37°C.
4. Pipette 2 ml sperm washing medium into each test tube and mix.
5. Centrifuge at 600*g* for 5 - 10 minutes.
6. Discard supernatant. Resuspend sperm pellet in 2 ml sperm washing medium.
7. Centrifuge at 600*g* for 5 - 10 minutes.
8. Discard supernatant. Resuspend sperm pellet in about 250 ul sperm washing medium.
9. Pipette 5 ul of this sperm suspension onto a prewarmed glass slide.
10. Pipette 5 ul of the washed Anti-Ig(H&L) Beads onto the sperm suspension. Use the pipette tip to mix the suspension and Anti-Ig(H&L) Beads thoroughly.
11. Place a coverslip on top of the mixture.
12. After 2 - 5 minutes examine the slide using a microscope.
13. Count 100 free-swimming sperm and determine if and where any beads are bound to the surface of the sperm.

Calculation of Percent Total Binding:

Count moving sperm and score as follows:
free = no beads attached
head = bead(s) attached to sperm head
midpiece = bead(s) attached to sperm midpiece
tail = bead(s) attached along tail length
entire = beads attached to more than one location on sperm

Calculate the percent total binding of beads as the sum of all percent bound beads from the various locations on the sperm surface per 100 sperm.

$$\% \text{ total binding} = \frac{\text{No. sperm with bound beads}}{\text{Total no. sperm counted}} \times 100\%$$

Example: At 400X the following data were obtained for an unknown sperm sample mixed with Anti-Ig(H&L) Beads:

- free = 50
- head = 12
- midpiece = 10
- tail = 18
- entire = 10

Applying the formula:

$$\frac{12 + 10 + 18 + 10}{100} \times 100\% = 50\% \text{ total binding of Anti-Ig(H\&L)}$$

Selected Reference:

World Health Organization. 1999. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Cambridge University Press. Fourth Edition.

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Bead Method for the Detection of Sperm-Reactive Antibodies

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In the *Direct* ImmunoSpheres®, live motile sperm are mixed with a suspension of bead reagent. As the sperm swim through the bead suspension, the beads will bind to the sperm if antibodies are present on the sperm.

In the *Indirect* ImmunoSpheres®, live motile sperm are incubated with diluted serum. Any antibodies to sperm present in the serum will bind to the sperm. Then unbound antibodies and serum proteins are washed away.

In the next step, these sperm are mixed with a suspension of bead reagent. The protocol proceeds as in the *Direct* ImmunoSpheres®.

Reagents:

Anti-IgA Beads: 0.8 ml red latex beads coated with (goat) anti-human IgA in protein buffer with 0.1% sodium azide.
Anti-IgG Beads: 0.8 ml blue latex beads coated with (goat) anti-human IgG in protein buffer with 0.1% sodium azide.
Anti-IgM Beads: 0.8 ml green latex beads coated with (goat) anti-human IgM in protein buffer with 0.1% sodium azide.

Materials Required But Not Provided:

1. Sperm washing medium containing 1 - 2% bovine serum albumin.
2. Positive and negative serum controls.
3. Centrifuge capable of 1000*g*.
4. 37ºC incubator.
5. Conical centrifuge tubes and rack.
6. Pipettors and tips.
7. Glass slides and coverslips.
8. Sperm counting chamber.
9. 56ºC incubator.
10. Bright-field microscope with 100X - 400X magnification.
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Storage and Stability:

Store the reagents at 4ºC. They can be used until the expiration date on each label.

All Beads should be stored in an upright position.

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Limitations:

Direct ImmunoSpheres®: Sperm with a motility of less than 5 million/ml cannot be used in this test. Indirect ImmunoSpheres®. At least 50 million motile sperm/ml are needed.

Preparation for Direct ImmunoSpheres®:

1. Bring all reagents to room temperature.
2. Warm sperm washing medium to 37ºC. *Caution*: Do not use medium containing human serum albumin.
3. Preparation of Beads:
   - 3.1. Gently swirl the vial containing the Anti-IgA Beads, avoiding foaming, to resuspend the Beads.
   - 3.2. Remove an aliquot (use 10 ul for each sample you will be testing) to a conical centrifuge tube.
   - 3.3. Add 2 -3 ml sperm washing medium.
   - 3.4. Centrifuge at 1000*g* for 5 -10 minutes, remove supernatant.
   - 3.5. Add 2 -3 ml sperm washing medium.
   - 3.6. Centrifuge at 1000*g* for 5 -10 minutes, remove supernatant and resuspend bead pellet to its original aliquot volume.
   - 3.7. Store unused washed Anti-IgA Beads at 4ºC for up to 3 days.
   - 3.8. Repeat steps 3.1 to 3.7 using Anti-IgG Beads.
   - 3.9. Repeat steps 3.1 to 3.7 using Anti-IgM Beads.
4. Semen preparation:
   - 4.1. Allow semen sample to liquify.
   - 4.2. Add sufficient sperm washing medium to equal twice the volume of the semen sample and mix. For example, for 2 ml semen, add 4 ml sperm washing medium.
   - 4.3. Centrifuge at 600*g* for 5 - 10 minutes, remove supernatant, and resuspend sperm pellet in about 3 ml sperm washing medium.
   - 4.4. Centrifuge at 600*g* for 5 - 10 minutes, remove supernatant, and resuspend sperm pellet in a small volume of sperm washing medium.
   - 4.5. Count sperm and determine motility of washed sperm.
   - 4.6. Dilute sperm to give a final concentration of 10 million motile sperm/ml.

Procedure for Direct ImmunoSpheres® of Sperm:

1. Pipette 5 ul of the sperm suspension onto a prewarmed glass slide.
2. Pipette 5 ul of the washed Anti-IgA Beads onto the sperm suspension. Use the pipette tip to mix the suspension and Anti-IgA Beads thoroughly.
3. Place a coverslip on top of the mixture.
4. After 1 - 2 minutes examine the slide using a microscope.
5. Count 100 free-swimming sperm and determine if and where any beads are bound to the surface of the sperm.
6. Repeat steps 1 to 5 using the washed Anti-IgG Beads.
7. Repeat steps 1 to 5 using the washed Anti-IgM Beads.

Preparation for Indirect ImmunoSpheres®of Serum:

1. Bring all reagents to room temperature.
2. Warm sperm washing medium to 37ºC. Caution: Do not use medium containing human serum albumin.
3. Preparation of Beads:
   - 3.1. Gently swirl the vial containing the Anti-IgA Beads, avoiding foaming, to resuspend the beads.
   - 3.2. Remove an aliquot (use 10 µl for each sample you will be testing) to a conical centrifuge tube.
   - 3.3. Add about 10 ml sperm washing medium.
   - 3.4. Centrifuge at 1000*g* for 5 -10 minutes, remove supernatant.
   - 3.5. Add 2 -3 ml sperm washing medium.
   - 3.6. Centrifuge at 1000*g* for 5 -10 minutes, remove supernatant and resuspend bead pellet to its original aliquot volume.
   - 3.7. Store unused washed Anti-IgA Beads at 4ºC for up to 3 days.
   - 3.8. Repeat steps 3.1 to 3.7 using Anti-IgG Beads.
   - 3.9. Repeat steps 3.1 to 3.7 using Anti-IgM Beads.
4. Serum preparation: heat inactivate serum by incubating at 56ºC for 30 minutes.
5. Semen preparation:
   - 5.1. Allow semen sample to liquify.
   - 5.2. Add sufficient sperm washing medium to equal twice the volume of the semen sample and mix. For example, for 2 ml semen, add 4 ml sperm washing medium.
   - 5.3. Centrifuge at 600*g* for 5 - 10 minutes, remove supernatant, and resuspend sperm pellet in about 3 ml sperm washing medium.
   - 5.4. Centrifuge at 600*g* for 5 - 10 minutes, remove supernatant, and resuspend sperm pellet in a small volume of sperm washing medium.
   - 5.5. Count sperm and determine motility of washed sperm.
   - 5.6. Dilute sperm to give a final concentration of 50 million motile sperm/ml.

Procedure for Indirect ImmunoSpheres® of Serum:

1. Pipette 50 µl of the following into separate test tubes:
   a known positive control serum,
   a known negative control serum, and
   each unknown serum.
2. Pipette 400 ul of the sperm washing medium into each test tube.
3. Pipette 50 ul of the donor sperm suspension into each test tube. Mix gently. Cover each test tube and incubate 60 minutes at 37ºC.
4. Pipette 2 ml sperm washing medium into each test tube and mix.
5. Centrifuge at 600*g* for 5 - 10 minutes.
6. Discard supernatant. Resuspend sperm pellet in 2 ml sperm washing medium.
7. Centrifuge at 600*g* for 5 - 10 minutes.
8. Discard supernatant. Resuspend sperm pellet in about 250 ul sperm washing medium.
9. Pipette 5 ul of this sperm suspension onto a prewarmed glass slide.
10. Pipette 5 ul of the washed Anti-IgA Beads onto the sperm suspension. Use the pipette tip to mix the suspension and Anti-IgA Beads thoroughly.
11. Place a coverslip on top of the mixture.
12. After 1-2 minutes examine the slide using a microscope.
13. Count 100 free-swimming sperm and determine if and where any beads are bound to the surface of the sperm.
14. Repeat steps 9 to 13 using the washed Anti-IgG Beads.
15. Repeat steps 9 to 13 using the washed Anti-IgM Beads.

Calculation of Percent Total Binding:

- Count moving sperm and score as follows
- free = no beads attached
- head = bead(s) attached to sperm head
- midpiece = bead(s) attached to sperm midpiece
- tail = bead(s) attached along tail length
- entire = beads attached to more than one location on sperm

Calculate the percent total binding of beads as the sum of all percent bound beads from the various locations on the sperm surface per 100 sperm.

$$\% \text{ total binding} = \frac{\text{No. sperm with bound beads}}{\text{Total no. sperm counted}} \times 100\%$$

Example: At 400X the following data were obtained for an unknown sperm sample mixed with Anti-IgG Beads:

- free = 60
- head = 12
- midpiece = 0
- tail = 18
- entire = 10

Applying the formula:

$$\frac{12 + 0 + 18 + 10}{100} \times 100\% = 40\% \text{ total binding of Anti-IgG}$$

Selected References:

1. Centola GM, Andolina E, Deutsch A. 1997. Comparison of the immunobead binding test (IBT) and immunospheres (IS) assay for detecting serum antisperm antibodies. Am J Reprod Immunol. 37:300-3.
2. World Health Organization. 1999. *WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction.* Cambridge University Press. Fourth Edition.
3. Chamley LW, Clarke GN. 2007. Antisperm antibodies and conception. Semin Immunopathol. 29:129-184.

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