ab65339
Creatine Assay Kit (Colorimetric/Fluorometric)

Instructions for Use

For rapid, sensitive and accurate measurement of creatine levels in various samples.

This product is for research use only and is not intended for diagnostic use.

PLEASE NOTE: With the acquisition of BioVision by Abcam, we have made some changes to component names and packaging to better align with our global standards as we work towards environmental-friendly and efficient growth. You are receiving the same high-quality products as always, with no changes to specifications or protocols.

Version 4 Last Updated 7 June 2023

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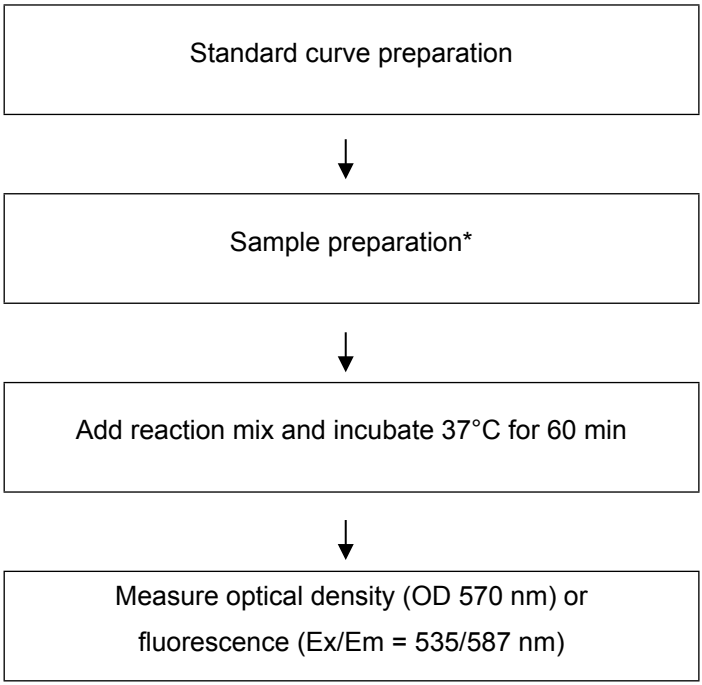
1. BACKGROUND

Creatine Assay Kit (Colorimetric/Fluorometric) (ab65339) provides an accurate, convenient measure of creatine in a variety of biological samples. In the assay, creatine is enzymatically converted to sarcosine which is then specifically oxidized to generate a product that converts a colorless probe to an intensely red color (λmax = 570nm), and highly fluorescent (Ex/Em = 538/587 nm) product. Creatine is therefore easily detected by either colorimetric or fluorometric methods. Detection range 0.001 – 10 mM Creatine.

Creatine is an endogenous compound whose function is to maintain a high ATP/ADP ratio, by way of its phosphorylated form and creatine kinase. Creatine supplementation has been used in the treatment of muscular, neurological and neurodegenerative diseases, as well as a sport performance enhancer. Detection of creatine level has wide applications in research and development.

2. ASSAY SUMMARY



*Samples might require deproteinization.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at -20ºC in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 5.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
Assay Buffer II/Creatine Assay Buffer	25 mL	-20°C	4°C; -20°C
OxiRed Probe/Creatine Probe	200 µL	-20°C	-20°C
Creatinase (lyophilized)	1 vial	-20°C	-20°C
Sarcosine Enzyme Mix/Creatine Enzyme Mix (lyophilized)	1 vial	-20°C	-20°C
Creatine Standard (10 µmol) (lyophilized)	1 vial	-20°C	-20°C

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- MilliQ water or other type of double distilled water (ddH_2O)
- PBS
- Microcentrifuge
- Pipettes and pipette tips
- Colorimetric or fluorescent microplate reader – equipped with filter for OD 570 nm or Ex/Em = 535/587 nm (respectively)
- 96 well plate: clear plates for colorimetric assay; black plates (clear bottoms) for fluorometric assay
- Heat block or water bath
- Dounce homogenizer or pestle (if using tissue)

For deproteinization step, additional reagents are required:

- Perchloric acid (PCA) 4M, ice cold
- Potassium Hydroxide (KOH) 2M
- 10 kD Spin Columns (ab93349) – for fluid samples, if not performing PCA precipitation

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Keep enzymes, heat labile components and samples on ice during the assay.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **Assay Buffer II/Creatine Assay Buffer:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C or -20°C.

9.2 **OxiRed Probe/Creatine Probe – in DMSO:**

9.3 Ready to use as supplied. Warm by placing in a 37°C bath for 1 – 5 minutes to thaw the DMSO solution before use. **NOTE: DMSO tends to be solid when stored at -20°C, even when left at room temperature, so it needs to melt for few minutes at 37°C.** Aliquot OxiRed Probe/probe so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light and moisture. Once OxiRed Probe/probe is thawed, use within two months. Keep on ice while in use.

9.4 **Creatinase:**

Reconstitute in 220 µL Assay Buffer II/Assay Buffer. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Freeze/thaw should be limited to one time. Store at -20°C. Keep on ice while in use.

9.5 **Sarcosine Enzyme Mix/Creatine Enzyme Mix:**

Reconstitute in 220 µL Assay Buffer II/Assay Buffer. Aliquot Sarcosine Enzyme Mix/enzyme mix so that you have enough volume to perform the desired number of assays. Freeze/thaw should be limited to one time. Store at -20°C. Keep on ice while in use.

9.6 **Creatine Standard:**

Reconstitute the Creatine Standard (10 µmol) in 100 µL of ddH_2O to generate a 100 nmol/µL standard stock solution. Pipette up and down to dissolve completely. Aliquot standard so that you have enough volume to perform the desired number of assays. Store at -20°C. Use within 2 months. Keep on ice while in use.

10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be used within 4 hours.

10.1 For the colorimetric assay:

10.1.1 Prepare 500 µL of 1 nmol/µL Creatine standard by diluting 5 µL of the reconstituted standard with 495 µL of Assay Buffer II/Assay Buffer.

10.1.2 Using 1 nmol/µL Creatine standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Standard (µL)	Assay Buffer II/Assay Buffer (µL)	Final volume standard in well (µL)	End [Creatine] in well
1	0	150	50	0 nmol/well
2	6	144	50	2 nmol/well
3	12	138	50	4 nmol/well
4	18	132	50	6 nmol/well
5	24	126	50	8 nmol/well
6	30	120	50	10 nmol/well

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).

10.2 **For the flurometric assay:**

10.2.1 Prepare a 1 nmol/µL standard as described in Section 10.1.1.

10.2.2 Prepare 500 µL of 0.1 nmol/µL Creatine standard by diluting 50 µL of 1 nmol/µL Standard with 450 µL of Assay Buffer II/Assay Buffer.

10.2.3 Using 0.1 nmol/µL standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Standard (µL)	Assay Buffer II/Assay Buffer (µL)	Final volume standard in well (µL)	End [Creatine] in well
1	0	150	50	0
2	6	144	50	0.2 nmol/well
3	12	138	50	0.4 nmol/well
4	18	132	50	0.6 nmol/well
5	24	126	50	0.8 nmol/well
6	30	120	50	1.0 nmol/well

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).

NOTE: *If your sample readings fall out the range of your fluorometric standard curve, you might need to adjust the dilutions and create a new standard curve. Alternatively, further dilute the standard.*

11. SAMPLE PREPARATION

General Sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step as well as the deproteinization step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1 Cell (adherent or suspension) samples:

11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = 2 x 10^6 cells).

11.1.2 Wash cells with cold PBS.

11.1.3 Resuspend cells in 100 µL of Assay Buffer II/Assay Buffer.

11.1.4 Homogenize cells quickly by pipetting up and down a few times. Incubate on ice 10 – 30 minutes.

11.1.5 Centrifuge sample for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.1.6 Collect supernatant and transfer to a clean tube.

11.1.7 Keep on ice.

11.1.8 Perform deproteinization step as described in section 11.4.

11.2 Tissue samples:

11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg).

11.2.2 Wash tissue in cold PBS.

11.2.3 Resuspend tissue in 100 µL of Assay Buffer II/Assay Buffer.

11.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes. Incubate on ice 10 – 30 minutes.

11.2.5 Centrifuge samples for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.2.6 Collect supernatant and transfer to a clean tube.

11.2.7 Keep on ice.

11.2.8 Perform deproteinization step as described in section 11.4.

11.3 **Plasma and Serum samples:**

Plasma and serum samples generally contain high amount of proteins, so they should be deproteinized as described in section 11.4.

Alternatively, you can use 10kD Spin column (ab93349) to deproteinize biological fluids.

11.4 **Deproteinization step:**

Prepare samples as specified in protocol. You should have a clear protein sample after homogenization and centrifugation. Keep your samples on ice.

11.4.1 Add ice cold PCA 4 M to a final concentration of 1 M in the homogenate solution and vortex briefly to mix well. *NOTE: high protein concentration samples might need more PCA*.

***NOTE**: high protein concentration samples might need more PCA.*

11.4.2 Incubate on ice for 5 minutes.

11.4.3 Centrifuge samples at 13,000 x g for 2 minutes at 4°C in a cold centrifuge and transfer supernatant to a fresh tube. Measure volume of supernatant.

11.4.4 Precipitate excess PCA by adding ice-cold 2 M KOH that equals 34% of the supernatant to your sample (for instance, 34 µL of 2 M KOH to 100 µL sample) and vortexing briefly. This will neutralize the sample and precipitate excess PCA.

11.4.5 After neutralization, it is very important that pH equals 6.5 – 8 (use pH paper to test 1 µL of sample). Any left over PCA

will interfere with the assay. If necessary, adjust pH with 0.1 M KOH.

11.4.6 Centrifuge at 13,000 x g for 15 minutes at 4°C and collect supernatant.

Samples are now deproteinized, neutralized and PCA has been removed. The samples are now ready to use in the assay.

Sample Recovery

The deproteinized samples will be diluted from the original concentration.

To calculate the dilution factor of your final sample, simply apply the following formula:

$$\% \text{ original concentration} = \frac{\text{Initial sample volume}}{\text{(initial sample volume + vol PCA + vol KOH)}} \text{X100}$$

NOTE: *We suggest using different volumes of sample to ensure readings are within the Standard Curve range.*

12. ASSAY PROCEDURE and DETECTION

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

12.1 **Set up Reaction wells:**

- Standard wells = 50 µL standard dilutions.
- Sample wells = 0 – 50 µL samples (adjust volume to 50 µL/well with Assay Buffer II/Assay Buffer).
- Background control sample wells= 0 – 50 µL samples (adjust volume to 50 µL/well with Assay Buffer II/Assay Buffer). ***NOTE:*** *for samples with high sarcosine content as it will generate background*.

12.2 **Reaction Mix (COLORIMETRIC ASSAY):**

Prepare 50 µL of Reaction Mix for each reaction

Component	Reaction Mix (µL)	Background Control Reaction Mix (µL)
Assay Buffer II/Creatine Assay Buffer	**44**	**46**
Creatinase	**2**	**0**
Sarcosine Enzyme Mix/Creatine Enzyme Mix	**2**	**2**
OxiRed Probe/Creatine Probe	**2**	**2**

Mix enough reagents for the number of assays (samples, standards and background control) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation:

X µL component x (Number samples + standards +1)

12.3 **Reaction Mix (FLUOROMETRIC ASSAY):**

Prepare 50 µL of Reaction Mix for each reaction

Component	Reaction Mix (µL)	Background Control Reaction Mix (µL)
Assay Buffer II/Creatine Assay Buffer	**45.6**	**47.6**
Creatinase	**2**	**0**
Sarcosine Enzyme Mix/Creatine Enzyme Mix	**2**	**2**
OxiRed Probe/Creatine Probe	**0.4**	**0.4**

**For fluorometric readings, using 0.4 µL/well of the OxiRed Probe/probe decreases the background readings, therefore increasing detection sensitivity.*

Mix enough reagents for the number of assays (samples, standards and background control) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation:

X µL component x (Number samples + Standards +1)

12.4 Add 50 µL of appropriate Reaction Mix to standard and sample wells.

12.5 Mix well. Incubate at 37°C for 60 minutes protected from light.

12.6 Measure output on a microplate reader.

- Colorimetric assay: measure OD 570 nm.
- Fluorometric assay: measure Ex/Em = 535/587 nm.

13. CALCULATIONS

- **Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.**
- **For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).**

13.1 Average the duplicate reading for each standard and sample.

13.2 If the sample background control is significant, then subtract the sample background control from sample reading.

13.3 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.4 Plot the corrected absorbance values for each standard as a function of the final concentration of creatine.

13.5 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).

13.6 Concentration of creatine (nmol/µL or mM) in the test samples is calculated as:

$$Creatine\ concentration = \left(\frac{A}{B}\right) * D$$

Where:

A = Amount of creatine in the sample well (nmol).

B = Sample volume added into the reaction well (µL).

D = Sample dilution factor.

Creatine molecular weight: 131.13 g/mol.

14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

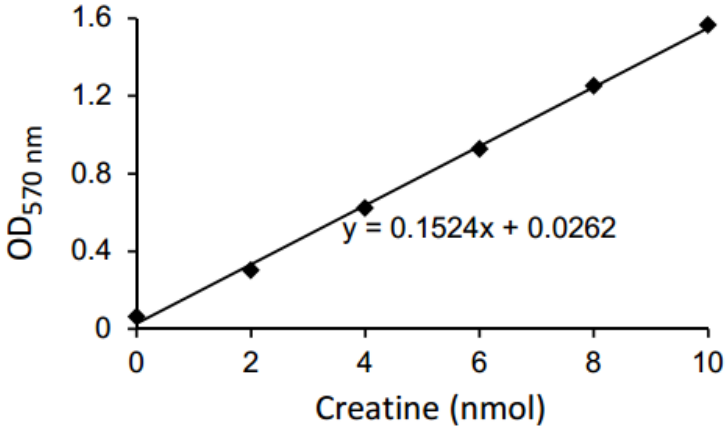


Figure 1. Typical sarcosine standard calibration curve using colorimetric reading.

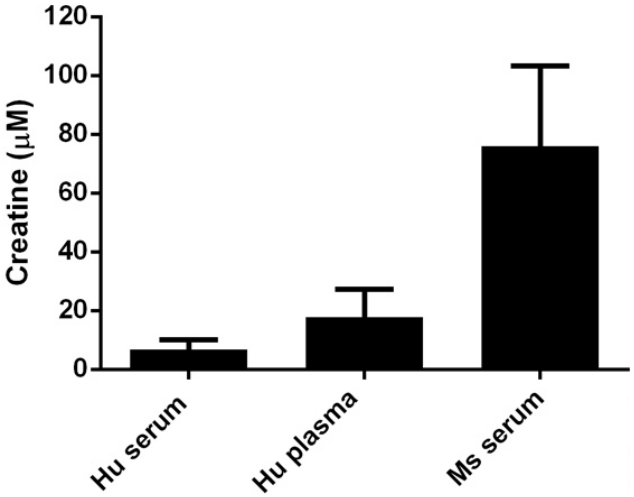


Figure 2: Creatine measured in various samples showing concentration (micromolar).

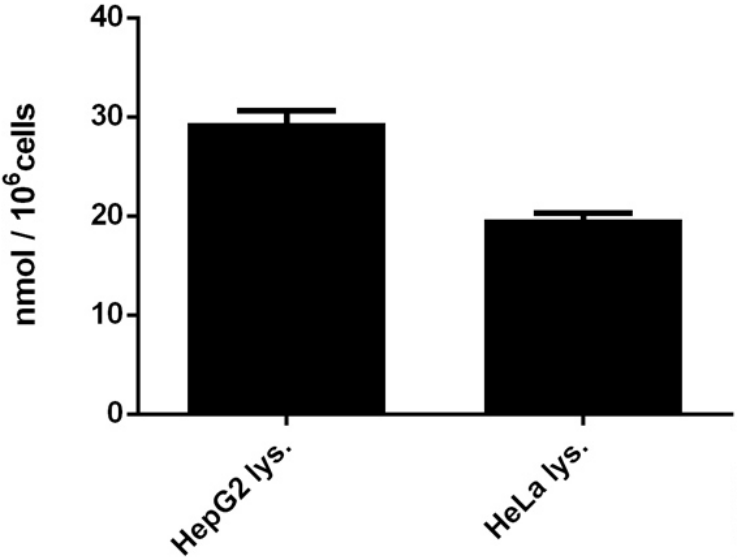


Figure 3: Creatine measured in cell lysates showing quantity (nmol) per 10^6 cells.

15. QUICK ASSAY PROCEDURE

***NOTE**: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.*

- Prepare standard, OxiRed Probe/probe, enzyme and Sarcosine Enzyme Mix/enzyme mix (aliquot if necessary); get equipment ready.
- Prepare appropriate standard curve for your detection method of choice (colorimetric or fluorometric).
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings), including deproteinization step.
- Set up plate for standard (50 µL), samples (50 µL) and background wells (50 µL).
- Prepare Creatine Reaction Mix (Number samples + background control + standards + 1).

Component	Colorimetric Reaction Mix (µL)	Background Control Reaction Mix (µL)
Assay Buffer II/Creatine Assay Buffer	**44**	**46**
Creatinase	**2**	**0**
Sarcosine Enzyme Mix/Creatine Enzyme Mix	**2**	**2**
OxiRed Probe/Creatine Probe	**2**	**2**

Component	Fluorometric Reaction Mix (µL)	Background Control Reaction Mix (µL)
Assay Buffer II/Creatine Assay Buffer	**45.6**	**47.6**
Creatinase	**2**	**0**
Sarcosine Enzyme Mix/Creatine Enzyme Mix	**2**	**2**
OxiRed	**0.4**	**0.4**

Probe/Creatine Probe		

- Add 50 µL of Reaction Mix to the standard, sample and background control wells.
- Incubate plate at 37°C 60 minutes protected from light.
- Measure plate at OD 570 nm for colorimetric assay or Ex/Em= 535/587 nm for fluorometric assay.

16. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use PCA precipitation protocol for deproteinization
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Lower/ Higher readings in samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

Problem	Cause	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions on protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

17. FAQ

What is the sensitivity of this assay and how can plasma/whole blood samples be processed for this assay?
The assay has a detection sensitivity of 0.5 mU/ml of glutathione peroxidase in samples.

Which protein assay is compatible with this kit?
We suggest you use a detergent compatible BCA assay kit: (ab102536).

What is the sample volume to be used with this kit for plasma samples from rat?
This depends on the amount of active CK enzyme in the sample. The sample volume per well would need to be optimized to make sure that the values obtained are within the linear range of the std. curve.

All the standards and the samples, including ones without creatine turned dark pink. What could be wrong?
If the OxiRed Probe/probe was exposed to light/air it might get oxidized and yield color.

Is it known if pyruvic acid, glucose, albumin, bilirubin and ascorbic acid interfere in this assay?
We are not aware of any interference by pyruvic acid, glucose, albumin, bilirubin or ascorbic acid. There might be cause-effect relationship between the creatine level in the sample and the concentration of these moieties but there is no reported interference with this assay per se that we are aware of. Sarcosine causes interference in this assay, which can be resolved by running side by side controls and subtracting the value from the results.

If there is no significant background, is it necessary to run background control for every sample?

Sarcosine creates background in this assay. If there is no significant background seen during the pilot assay, it is not essential to run background controls for every sample.

18. INTERFERENCES

These chemicals or biological materials will cause interferences in this assay causing compromised results or complete failure:

- Sarcosine.

19. **NOTES**

For all technical and commercial enquires please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)